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FERTILIZATION AND ARTIFICIAL ACTIVATION IN THE EGG OF THE SURF-CLAM, SPISULA SOLIDISSIMA[1]

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In fertilization the sperm generally performs two functions: activation and syngamy; these functions are experimentally separable. Under conditions where artificial parthenogenesis appears to involve the same initial sequence of developmental events that follow fertilization, it is believed that the stimulating agent somehow imitates the activating action of the sperm. Consequently, the term *parthenogenesis* (natural or artificial) which implies cleavage and later development, has been included under the wider term *activation*. (For reviews on activation and fertilization, see Loeb, 1913; Dalcq, 1928a; Tyler, 1941; Lillie, 1941; Runnström, 1949; Brachet, 1950, Chapter IV; and Heilbrunn, 1952, Chapter XL.) In recent years egg activation has been considered also as a specific case in the general phenomenon of excitation. It is generally true that activating agents for eggs also are stimulating agents for other kinds of cells. For this reason it is logical to extend the same concepts of excitation to egg cells that have been applied to stimulation and response in nerve, muscle, or protozoan cells. Furthermore, work done on various phases of egg activation complements that being done on other kinds of cells.

A study of excitation requires that a response be detectable soon after application of the stimulating agent. The surf-clam egg has a great advantage in this respect, for it contains a large germinal vesicle, the membrane of which breaks down readily upon stimulation. Thus the breakdown of the nuclear membrane can be taken as a criterion of response. The presence of polar bodies or of pronuclei at about 45 minutes after activation was taken as evidence that the cell was alive.

Previous studies on artificial parthenogenesis, especially those involving the eggs of echinoderms, often employed cleavage as a criterion of activation. However, in *Spisula* eggs and other eggs where fertilization occurs before the onset of maturation division, activation is not always followed by cleavage.

[1] Presented to the Faculty of the Graduate School of the University of Pennsylvania in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[2] Predoctoral Fellow, National Cancer Institute, U. S. Public Health Service.

The purpose of the present study has been to make a preliminary survey of fertilization and artificial activation in the surf-clam egg. Wherever possible, the approach has been from the standpoint of similarities and differences in the mechanisms involved in fertilization and artificial activation. Because *Spisula* eggs have been little used in previous studies of this kind, it was considered desirable to make observations on normal and parthenogenetic development, and also to repeat experiments performed by others on different eggs in order to establish a baseline for further work on the mechanism of activation.

Schechter (1941) described a method for obtaining gametes from surf-clams, and also the structural changes which occur in aging eggs. His paper also contains some observations on normal early development. The clam used by Schechter was *Spisula solidissima* (Dillwyn), although it has commonly been called *Mactra*. Kostanecki (1908) described artificial parthenogenesis in the eggs of *Mactra stultorum* and *M. helvacea;* these eggs were obtained from animals collected at Naples, Italy. The eggs of the species of *Mactra* studied by Kostanecki appear to be quite different in many respects from those of *Spisula solidissima*.

I wish to express my gratitude to Dr. L. V. Heilbrunn, under whose friendly guidance these studies were carried out, for helpful advice on experiments and assistance in the preparation of the manuscript. I wish also to thank Dr. H. G. Borei for his helpful criticisms of the manuscript. Thanks are also due to Dr. S. Inoué for the use of his polarization microscope and air-turbine centrifuge. This study was greatly aided through the kind cooperation of Mr. and Mrs. P. Breslin and Mr. F. Matthews of Belmar, New Jersey, in obtaining surf-clams and sea water.

Material and Methods

A. Material

Along the New Jersey coast, eggs of the surf-clam can readily be obtained at any time from early spring until late autumn. At Woods Hole, Massachusetts, the season is shorter, beginning late in May and extending into September. At Woods Hole there seems to be a period of one or two weeks in the middle of the season when gametes are scarce; this period often coincides with periods of excessive summer heat. The spawning of surf-clams can be induced during the winter months by warming them gradually and feeding them plankton over a period of about two weeks (Loosanoff and Davis, 1950).

For the experiments at the Zoological Laboratory, University of Pennsylvania, surf-clams were transported from a float in the channel of the Shark River Inlet in Belmar, New Jersey to Philadelphia, where they were maintained under aeration at about 14° C. in a constant temperature bath for one to three weeks. Four clams about four inches in length can be kept for a period of five to six days in one half gallon of sea water in a jar. It is particularly important to evacuate any air trapped in the gill chamber of the clams; otherwise death will result in a short time. This may be done by holding and gently squeezing each clam under water with its siphons facing upwards until all air bubbles have emerged. Natural sea water was employed in maintaining clams, and artificial sea water of the same salinity was used in all experiments with eggs in Philadelphia. In Woods Hole,

filtered sea water or artificial sea water could be used for the experiments; clams were obtained from Barnstable and kept in tanks of running sea water.

1. *Determination of sex*

Dr. L. Thomas, in work done at this laboratory, found that by inserting a medium-gauge hypodermic needle between the valves, near the hinge into the visceral mass, gametes could be drawn out for microscopic examination. This procedure permits separation of the sexes if desired, saves time and material, and could also be used for obtaining small amounts of gametes.

2. *Obtaining gametes*

Large numbers of gametes are obtained by breaking the shell near the umbo, cutting the adductor muscles and removing the visceral mass intact. The gills, mantle, and heart are trimmed away to expose the gonad, which then is excised. A large portion of the gonad lies deep within the visceral mass toward the foot. An excised ovary is cut up in a small volume of sea water and strained through a pad of cheesecloth into a large beaker containing filtered sea water. The eggs are allowed to settle to the bottom and the supernatant fluid is siphoned off. This washing process is repeated at least three times (Allen, 1951a). Thorough washing removes most of the blood, cellular debris from the ovary, and fertilizin. The latter substance has an adverse effect on fertilization in this species (Allen, 1951c). Excised testes are stored in the refrigerator until needed. During this storage period (up to six hours) a milky fluid containing sperm flows out of the gonad. This sperm suspension ("dry sperm") may be drawn readily into calibrated pipettes, permitting an accurate measurement of sperm concentrations. Precise measurements of sperm concentrations are required for some aspects of work involving fertilization.

3. *Structure of the unfertilized egg*

The unfertilized *Spisula* egg has a diameter of 56 microns, measuring from inside the vitelline membrane. The jelly layer, which is about two microns thick, is thinner than that of most marine eggs. It can usually be observed only after the addition of a dye (such as toluidine blue or Janus green B) or as an invisible barrier separating crowded eggs. The tough, easily visible vitelline membrane surrounds a thin, clear layer which is optically empty under bright field illumination, but negatively birefringent in the radial axis of the egg. Beneath the thin, clear layer is a differentiated cortex containing large granules which fail to move when forces of 200,000 × gravity are applied with a Beams-type, air-turbine centrifuge. The cytoplasm is moderately granular and about 11% of its volume is occupied by fat-containing granules (centrifuge experiments). Centrifugation stratifies these fat granules centripetally in a dense layer long before the heavy granules begin to collect at the opposite end of the cell. The properties of the cytoplasm are such that movement of the nucleus by centrifugal force produces a ring of negative strain birefringence in the region of the cytoplasm through which the nucleus has passed (Allen, 1952a). The germinal vesicle is about 31 microns in diameter and is somewhat eccentric. In life the nuclear membrane exhibits

weak negative radial birefringence; when fixed, it shows, distributed along its inner surface, the entire chromosome complement except that chromosome which is attached to the nucleolus. The chromosomes are minute tetrads. The nucleolus is double (amphinucleolus), consisting of a transparent nucleolus and an opaque nucleolinus (see Plate I, Fig. E.). These structures are approximately 11 and 3 microns in diameter, respectively. Similar double nucleoli have been seen in many other marine eggs in the oöcyte stage.

Freshly shed eggs are irregular in outline. However, by the time washing has been completed, they usually become spherical. Presumably this irregularity in shape is due to compression in the ovary.

4. *Fertilization and early development at 21° C.*

a. *Optimal conditions for fertilization.* As mentioned earlier (see also Allen, 1951a), it is important to wash Spisula eggs several times with filtered sea water before insemination. Failure to wash sufficiently (*i.e.*, to remove blood and fertilizin, etc.) will result in low percentages of fertilization and delayed or inhibited cleavage. Two other important considerations are egg and sperm densities. Stender dishes containing 10 ml. of sea water were placed in a constant temperature bath. One or two drops of washed concentrated eggs were placed in the dish, so that their number was approximately 200–300 per ml. Immediately, 0.05 ml. of a fresh 1 : 500 dilution of "dry sperm" in filtered sea water was added to each dish. The sperm density varies considerably and should be assayed in a counting chamber for accurate work. The final density should be corrected to about 10^8 cells/ml.

The time taken for penetration of the sperm into different eggs in a population can be inferred from the sigmoid curves obtained by plotting counts of germinal vesicle breakdown against time after insemination. Fifty per cent nuclear breakdown occurs at about 9½ minutes after insemination; the spread of the curve is about 2–2½ minutes. As the time lag between sperm penetration and nuclear breakdown is the same in every egg, this curve indicates that all of the eggs are probably fertilized within 2–2½ minutes after insemination, depending on the chance meetings of eggs and sperm in the suspension. Furthermore, increasing the sperm concentration increases the slope of the sigmoid curve, but high concentrations also tend to produce polyspermy. The slope of the curve often permits detection of lots of poor eggs or sperm.

b. *Normal development at 21° C.* Soon after fertilization some eggs exhibit a small indentation at one point on the surface. This is more pronounced in some lots of eggs than in others. At best it is seen in about a quarter of the eggs in a microscopic field, but as in ordinary microscopic observation only a portion of the egg surface can be observed at any time, it is likely that most or perhaps all of the eggs show this irregularity. Almost simultaneously, the vitelline membrane elevates gradually from the surface of the egg. This process differs from the membrane elevation of echinoderm eggs, for the cortical granules, which appear to be so important in the membrane elevation of the sea urchin egg, do not appear to participate in the elevation of the *Spisula* egg membrane. The cortical granules persist after fertilization and throughout early cleavages.

Six or seven minutes after insemination [3] the nucleus begins to change; the transparent nucleolus dissolves and releases its substances into the nucleoplasm. The nucleolinus comes to lie free in the nucleus, and remains in the vicinity of the forming maturation spindle even after the disappearance of the nuclear membrane. Observations on fixed material indicate a spindle-forming role for the nucleolinus (Allen, 1951b).

The first meiotic metaphase lasts from 13 to 26 minutes after insemination. During this time, the spindle migrates from the center to the periphery of the egg. The first polar body is extruded at 29 minutes and its position establishes the animal pole of the egg. At this time the animal pole flattens slightly, then rounds up again at 35 minutes. The second polar body appears 39 minutes after fertilization. There follows a second brief flattening before the male pronucleus becomes visible at 50 minutes. Within another minute the female pronucleus also appears, and the two nuclei swell almost simultaneously, to a diameter of 11–12 microns before they merge and break down, giving rise to a clear region in the center of the cell. At 69 minutes the eggs elongate parallel to the long axis of the cleavage spindle. Formation of the cleavage furrow begins at 71 minutes, and is complete by 74 minutes. The nucleus re-forms in the (smaller) AB cell at 77 minutes and in the (larger) CD cell at 82 minutes after insemination. At 89 minutes both nuclei disappear again almost simultaneously in preparation for the second division at 99 minutes. The second cleavage divides the A and B cells equally, but the C cell is smaller than the D cell. Further cell divisions are more rapid and somewhat difficult to observe in living material. Rotating blastulae are seen usually within four or five hours after insemination; swimming larvae within a day. Veliger larvae will live at least three weeks in artificial sea water without feeding. (For early stages of normal development, see Plates I and II.)

c. *Abnormalities.* Polyspermic eggs usually fail to cleave; sometimes, however, they divide into blastomeres of equal size, or develop into abnormal larvae. Presence of more than two pronuclei in fertilized eggs is an indication of polyspermy.

Occasionally the cytoplasm of fertilized eggs becomes dark in color after nuclear breakdown. The cause of this abnormality may be sea water impurities, unripe gametes or unhealthy clams. Lots of clams with eggs showing this dark cytolysis often exhibit high percentages of spontaneous germinal vesicle breakdown after about an hour.

Aging in *Spisula* eggs leads to two obvious changes: first, there often appear deep indentations on the surface of the eggs (Schechter, 1941); such eggs can usually be fertilized normally if not too old. Second, the nucleus loses some of its normal transparency. Although these changes are not significant with respect to development, they should be recognized as a factor possibly influencing the physiology of the egg (see Scott and Hayward, 1950).

An occasional lot of eggs shows clumping of the cytoplasmic granules. Such eggs were discarded although it was observed that they were often able to develop normally.

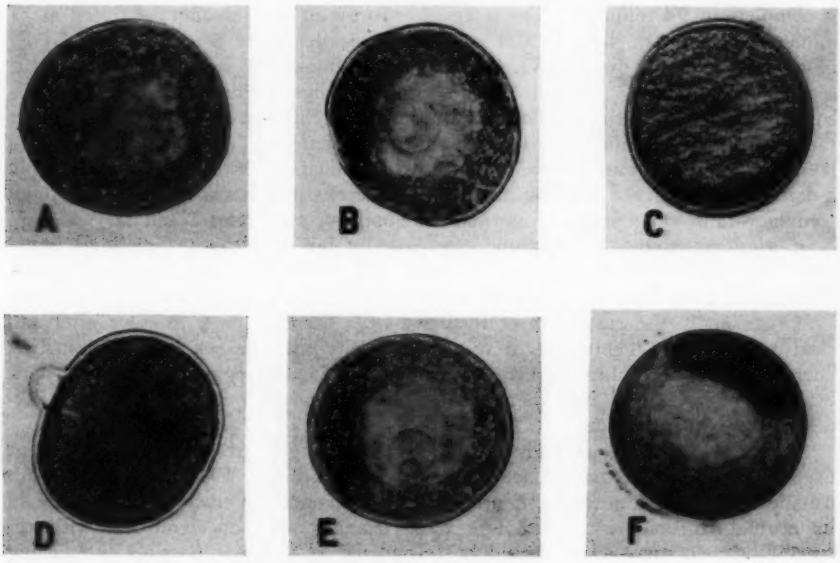
Under-ripe eggs often stick together in groups. This kind of agglutination

[3] All time figures given in this section refer to the time at which 50% of the eggs exhibit the particular phenomenon. This time is accurate to within a minute at constant temperature if egg batches from different females are compared under similar conditions. Under optimal conditions the spread of the points for eggs from a single female is about two minutes or less.

means that the jelly has not completely formed and is sticky. Such eggs are not suitable for experimental work, even though they may be fertilizable.

d. *Development of artificially activated eggs.* As far as could be determined, the early stages following artificial activation up to and including the breakdown of the nuclear membrane are identical with the early stages following fertilization. Furthermore, activated eggs usually form at least one polar body, often two; but with the methods tried so far, cleavage is rare. After one or two hours, activated eggs often exhibit from one to several nuclei, a condition highly suggestive of polyspermy. Such a multinucleate condition apparently comes about through a failure of the egg to form a second polar body; the second nucleus is retained in place of the male pronucleus. A similar situation was reported by Morris (1917) in the *Cumingia* egg, and by Kostanecki (1908) in *Mactra.* These nuclei sometimes undergo an abnormal mitotic reproduction without division of the cell; multipolar spindles are sometimes seen.

PLATE I. *Spisula solidissima* eggs



A. Unfertilized egg showing intact germinal vesicle.

B. Unfertilized egg shown 20 seconds after stimulation by isotonic sodium chloride in the presence of traces of calcium.

C. Egg 10 minutes after insemination showing the clear zone left after the dissolution of the germinal vesicle membrane.

D. Egg 30 minutes after insemination showing the first polar body; note the slightly elevated membrane (compare with B).

E. Unfertilized egg showing the double nucleolus in sharp focus.

F. A centrifuged egg showing the layer of light fat granules; note the sperm adhering to the surface of the jelly layer.

B. *Methods*

All experiments on artificial activation were carried out with widely dispersed eggs in order to eliminate crowding as a factor in excitation. A constant volume (10 ml.) of sea water or other medium was used throughout. Artificial activation experiments were carried out for the most part in petri dishes 5–6 cm. in diameter; water loss from evaporation was negligible. Experiments involving ether were carried out in stoppered vials. Since the threshold of stimulation depends on the temperature, this factor had to be controlled. For ordinary observations, room temperature (around 21 degrees) was sufficiently constant to eliminate errors from this source. For more exacting experiments a constant temperature water bath set at 21° (± 0.05°) was used.

The source of ultraviolet irradiation used in activation experiments was a Hanovia mercury arc lamp (Model number 7420) at a target distance of 35 cm. delivering approximately 4500 microwatts per square centimeter.

All solutions used in artificial activation experiments were isotonic to sea water with the exception of the mixtures used in osmotic stimulation. The exact isotonic concentration of sucrose used in centrifugation experiments could not be determined because the eggs always became very irregular in this medium. Unless stated otherwise, the pH of all solutions used was adjusted to 8.0–8.2 with 0.5 N NaOH. Mixtures of solutions and sea water are referred to as percentage (V/V) of isotonic solution in sea water (*e.g.*, "5% KCl-SW" means 5 volumes isotonic KCl and 95 volumes of sea water). In the figures, these percentage expressions have been converted to millimols of cation per liter of solution, since the concentrations of the other ions remain essentially constant.

Isotonic artificial sea water solutions were made up according to the formulas presented below, which were recalculated and modified after Lyman and Fleming (1940). Artificial sea water solutions lacking various of the cations were also used. For unbuffered solutions, bicarbonate was omitted. All solutions were prepared from water distilled in an all-Pyrex apparatus containing potassium permanganate and phosphoric acid.

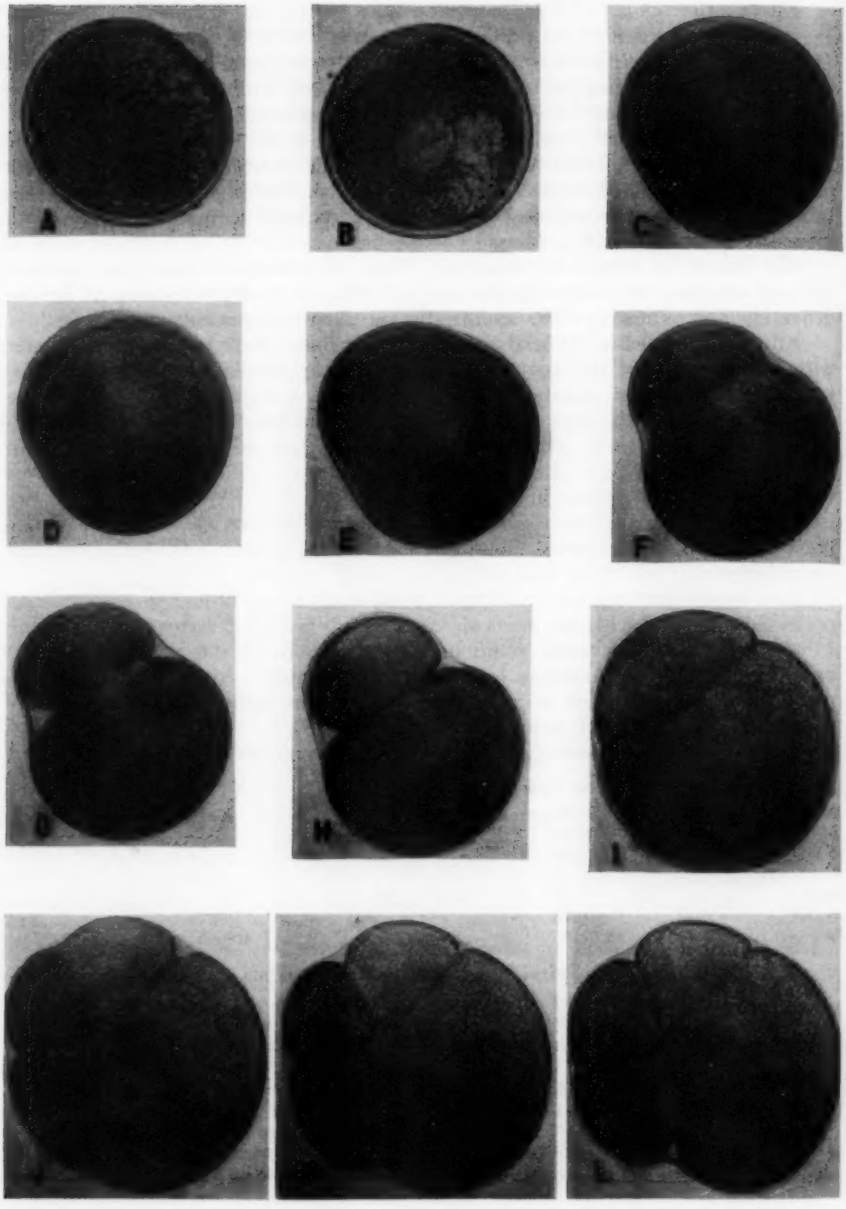
Formulas for approximately 4 liters of artificial sea water:

Substance	Sea water	Ca-Free	K-Free	Mg-Free
NaCl	93.90 grams	93.90	93.90	93.90
$MgCl_2$ ($6H_2O$)	42.35	42.35	42.35	—
Na_2SO_4	15.65	15.65	15.65	15.65
$CaCl_2$ ($2H_2O$)	6.10	—	6.10	6.10
KCl	2.65	2.65	—	2.65
$NaHCO_3$	0.76	0.76	0.76	0.76
Water	4076.0	3951.0	4003.0	3253.0

Isotonic solutions for Spisula eggs (from measurements of egg diameters):

Substance	Molar concentration
NaCl	0.50
KCl	0.52
$CaCl_2(2H_2O)$	0.35
$MgCl_2(6H_2O)$	0.34
Sucrose	0.73 (approximate)
Urea	0.96
Na citrate	0.35

PLATE II. *Spisula solidissima* eggs



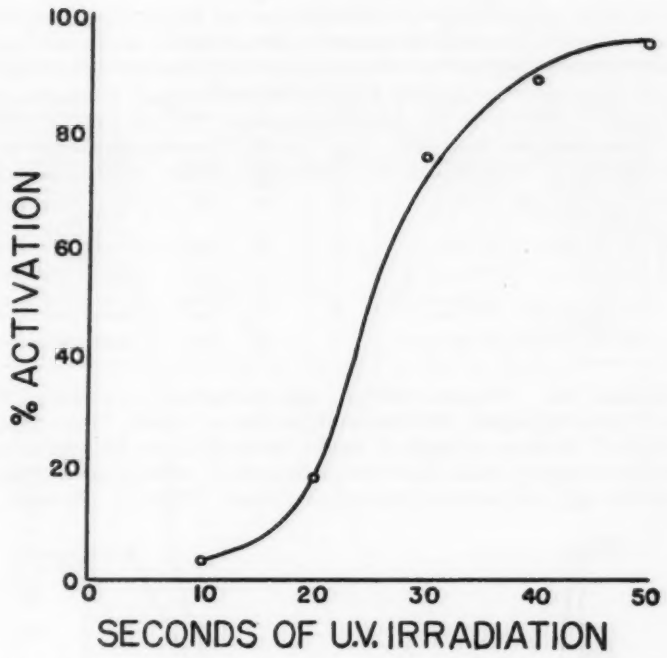


FIGURE 1. A typical population curve for a lot of eggs, obtained by plotting excitability against duration of exposure to the stimulating agent.

A sea water mixture containing only the chloride salts can be made by mixing isotonic chloride solutions in the following proportions: NaCl, 100 parts; KCl, 2.65; $CaCl_2$, 2.42; $MgCl_2$, 12.10.

RESULTS OF EXPERIMENTS

A. Artificial activation

Methods of activation were standardized to make possible a study of the influence on excitation of separate environmental factors and other agents. For this purpose curves were plotted for percentage of activation as a function of exposure time to the stimulating agent (usually ultraviolet light). The curve obtained in all cases was essentially one representing the distribution of excitabilities of individual eggs of a population. The shape and slope of the curve varied to some extent

A. Egg 43 minutes after insemination; note polar and clear region containing the spindle remnant.

B. The same egg 51 minutes after insemination; showing both pronuclei.

C–H. Photographs of a single egg at 21° C. taken at half-minute intervals from 71 minutes (C) to 73½ minutes (H).

I–L. Photographs taken of the same egg slightly compressed by a cover slide in order to show cleavage details. The time intervals between photographs was one-half minute, beginning at 93 minutes (I) and ending at 94½ minutes (L).

TABLE I

Artificial activation in Spisula eggs

Type of stimulation	Dose or concentration	Exposure time	No. of experiments	Maximum % activation	Reproducibility	Character of shape changes induced
U.V. light	4500 μWatts/cm.^{2}	15 sec.–1 min.	116	100	excellent	Slight wrinkles at surface
Excess K^+	4–6% KCl-SW	4+ min.	69	100	good	Slight wrinkles at surface
K-Free SW	100%	1–4 min.	8	100	good	Slight wrinkles at surface
NH_3	10^{-4}–10^{-3} *M*	1–4 min.	12	100	good	Slight wrinkles at surface
Hypertonicity	100 ml. SW + 30–50 ml. 2.5 N NaCl	1–5 min.	15	100	fair	Deep indentations into the cytoplasm
Hypotonicity	10–20% SW	1–3 min.	78	100	good	Deep indentations on return to sea water
Heat	25–40° C.	10 sec.–15 min.	8	10	poor	Deep indentations (prolonged but reversible)
Cold	0–5° C.	10 sec.–15 min.	4	10	poor	Deep indentations
Urea	1–100% isotonic urea-SW	1–10 min.	10	5	poor	Sudden deep indentations
Protamine	50–200 mg. %-SW	30 min.–4 hrs.	6	100	fair	Shallow indentations

among different lots. Whenever difficulty was encountered in obtaining 95–100% activation by artificial agents, fertilization of the lot was usually found to be equally poor. Figure 1 shows an example of such a threshold curve for a lot of eggs.

A number of agents which have been employed in artificial parthenogenesis of various marine eggs will activate the eggs of *Spisula* (Table I). In most cases, it

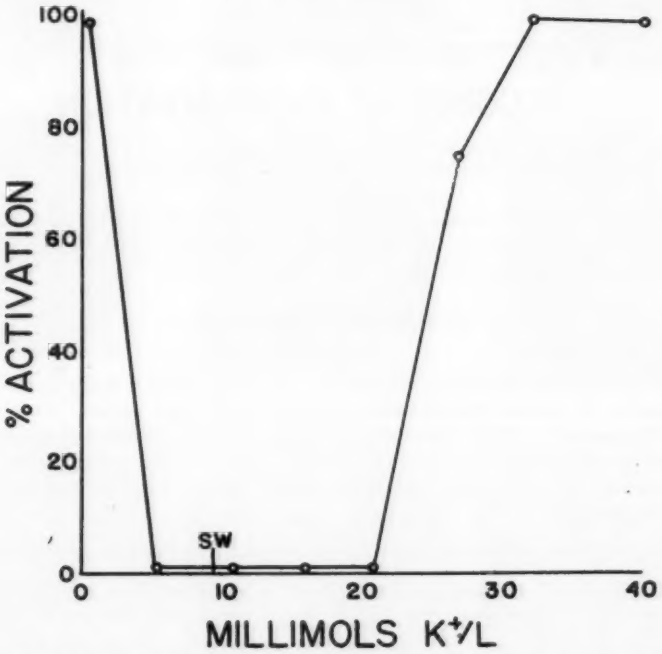


FIGURE 2. The activating effect of potassium ions above and below the concentration found in normal sea water.

was found possible to adjust the concentration of the activating agent so that only short exposure times were required for complete or nearly complete activation. Only in the case of excess potassium ions and of protamine (clupein) was it necessary to expose for longer than one to three minutes in order to obtain a result. Of the agents listed in Table I, only heat, cold and urea were distinctly unsatisfactory as activating agents for *Spisula* eggs. They are listed, however, for their interesting property of causing pronounced changes of shape in the eggs.

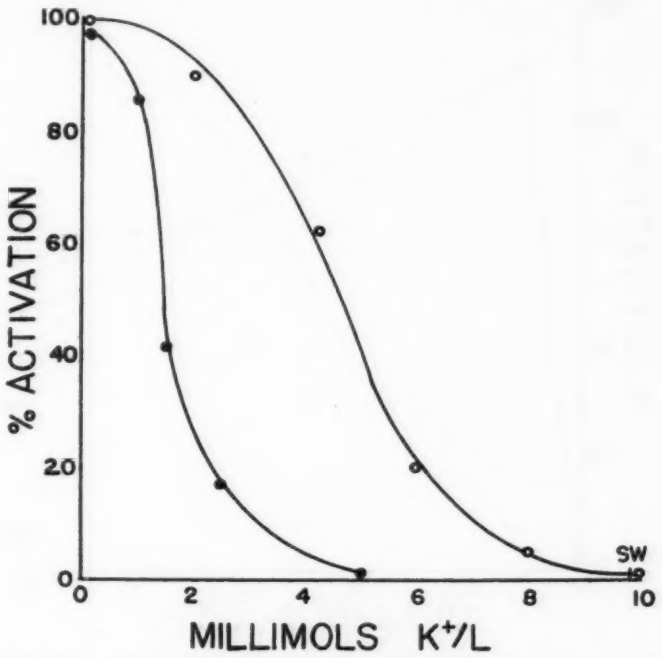


FIGURE 3. Curves showing (o) synergistic effect of low potassium concentrations in sea water and sub-threshold ultraviolet light stimulation, and (●) the activating effect of low potassium concentrations alone.

B. Changes in excitability following alteration of the medium

Experiments were carried out to determine the influence of changes in the environment on excitation, using an excitability curve (such as Fig. 1) as a guide to dosage. The concentration of each ion (except sodium) was varied while keeping tonicity and pH constant.

It was important to know how long eggs must remain in contact with a new medium before a change in excitability is manifested. The new environment causes the same degree of effect on the response whether the eggs are placed in it before, or up to four minutes after application of the activating agent. For this reason, in the experiments reported here, eggs were first stimulated by a dosage

of ultraviolet light (determined by a threshold curve) and then immediately transferred to separate dishes containing different amounts of the ion or other substance under consideration.

Even small increases or decreases in potassium concentration cause increased excitability. Both excess potassium and potassium-free sea water are excellent activating agents (Fig. 2). Figure 3 shows the effect of low potassium concentrations alone and in synergism with ultraviolet light.

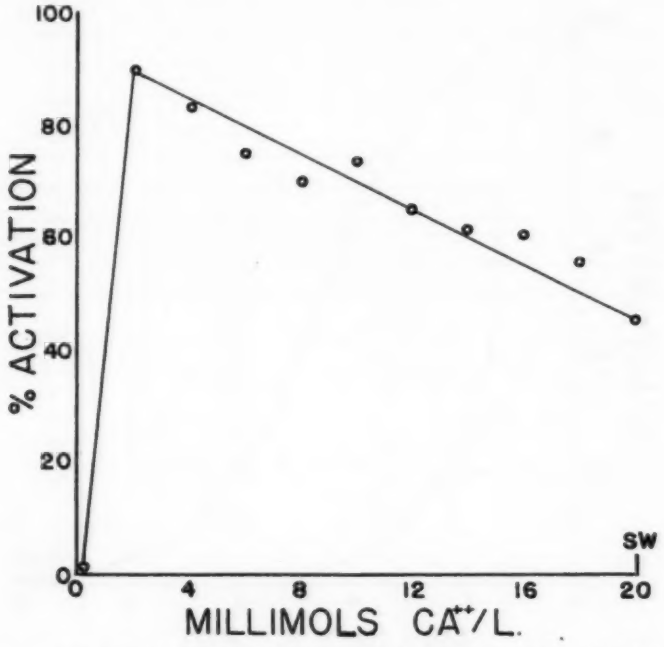


FIGURE 4. The effect of reduced calcium concentration on excitability (ultraviolet light stimulation). Dose of irradiation chosen to give 50% activation in normal sea water.

It was not possible to demonstrate an activating effect of any concentration of calcium. Calcium, however, apparently does enter and leave the egg, at least the cortical region, because even a slight alteration in calcium content of the medium causes a change in threshold to ultraviolet light stimulation (Fig. 4). Decreasing the calcium concentration results in a marked increase in excitability; a minimum amount of calcium is required for any excitation, however (about 5×10^{-4} M). Excess calcium ions reduce excitability. These effects are reversible.

If irradiation of the eggs by ultraviolet light in the absence of calcium is followed by transfer of the eggs into normal sea water, activation results. The percentage of activation is an inverse function of the time elapsed between the onset of irradiation and the transfer to the calcium-containing medium (Fig. 5). Other activating agents failed to give activation under these circumstances.

The effects produced by magnesium are quite similar to those of calcium as far as threshold is concerned. However, magnesium is apparently not necessary for activation. Figure 6 shows that excitability is high at low concentrations of magnesium and low when excess magnesium has been added.

Fertilization seems to be unaffected by changes in pH between 7 and 9 under conditions where ample sperm are present. At pH values below 7, the percentage of eggs activated by the sperm decreases rapidly. Below pH 5.0–5.2

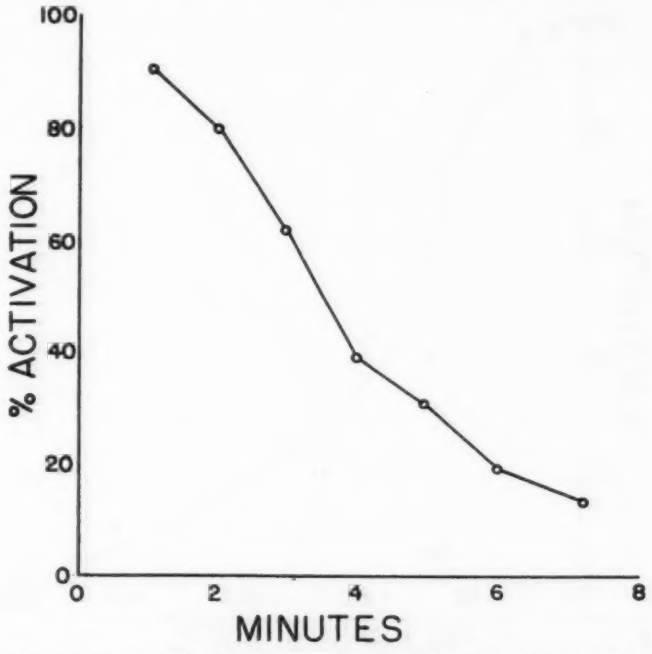


FIGURE 5. Activation obtained if eggs are irradiated in calcium-free sea water and subsequently transferred to normal sea water at the times indicated. Dose of irradiation chosen to give 100% activation in normal sea water.

there is usually no activation (Fig. 7). Using a strength of ultraviolet stimulation which at pH 8.0–8.2 activates 95–100% of the eggs, it is found that the pH at which total inhibition occurs is about the same as that at which fertilization is inhibited; see Figures 7 and 8. However, it appears that the shape of the pH-dependency curve differs for the three kinds of activation (sperm, ultraviolet light, and osmotic stimuli).

Since a rise in temperature accelerates the rate of many chemical reactions, it might be expected that heat would accelerate whatever reactions may be involved in excitation. In *Spisula* eggs, however, the opposite seems to be true; eggs stimulated at room temperature exhibit higher percentages of activation if trans-

ferred to sea water of lower temperature (Fig. 9). Similarly, eggs stimulated at low temperatures are inhibited if transferred to higher temperatures. On the other hand, previous exposure to heat (30–35° C.) or cold (0–5° C.) for a few minutes increases excitability when eggs are subsequently stimulated by ultraviolet light. It should be emphasized that heat and cold by themselves are not successful activating agents for *Spisula* eggs. These agents do, however, influence the

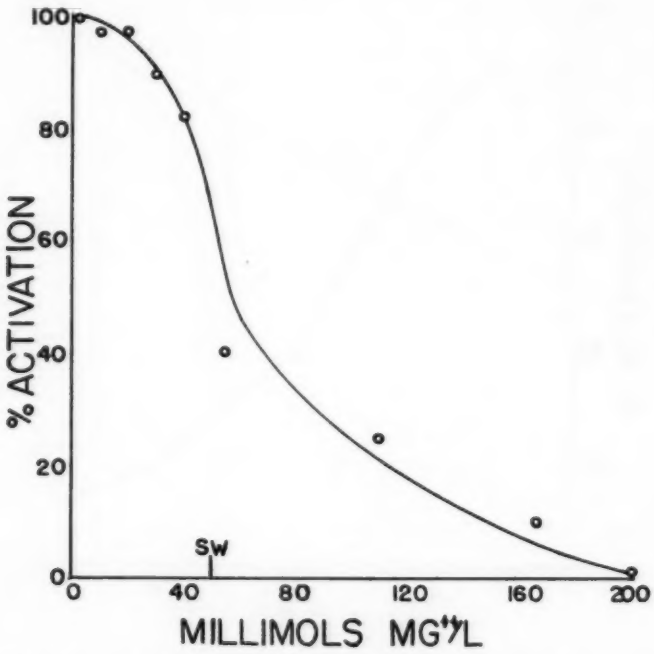


FIGURE 6. The influence of reduced and increased magnesium concentration on excitability. Dose of ultraviolet light chosen to give 50% activation in normal sea water.

response mechanism, as evidenced by their effects on the shape of the eggs and on excitability.

The anesthetics ethyl ether (Fig. 10) and ethyl urethane (Fig. 11) inhibit activation by sperm and decrease excitability for artificial activating agents. Chloroform causes extensive injury even at relatively low concentrations and therefore is not a good inhibitor for *Spisula* eggs. The egg jelly extracted with acid sea water and neutralized also exhibits a weak inhibitory influence on activation in sufficiently high concentrations.

Although normal fertilization causes only a slight and gradual membrane elevation, ultraviolet light in synergism with sea water of altered ionic constitution often causes a more pronounced elevation of the membranes. The monovalent cations are especially potent in this respect.

C. Duration of sensitivity to inhibitors

Fertilization in the presence of calcium could be reversed during the first four or five minutes of development by immersing the eggs in any of the following

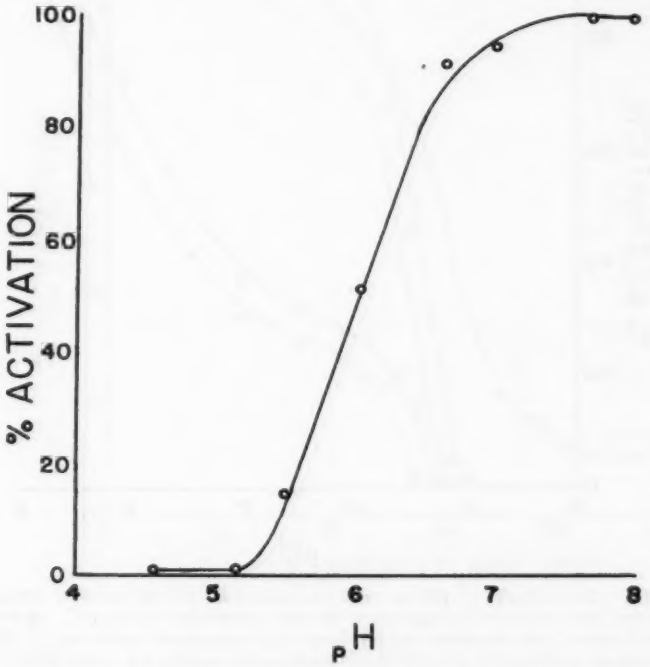


FIGURE 7. The influence of pH on fertilization (pH adjusted by adding HCl to bicarbonate-free sea water).

media: *a*, 10–20% isotonic sodium citrate [4] calcium-free sea water; *b*, acidified sea water (pH 5; made by adding 0.5 N HCl to unbuffered sea water), or *c*, 0.3–0.5% (by volume) ether in sea water. Developmental changes initiated during the first four or five minutes after fertilization could be reversed, because many of these eggs could be returned to normal sea water and activated a second time, this time by an artificial activating agent. These reactivated eggs often cleaved, in contrast

[4] Citrate is necessary to take care of the calcium carried over with the eggs; this concentration is well below the toxic level.

to the artificially activated ones which almost never cleave. Figure 12 defines the term "Stage I" which is the period during which eggs activated in normal sea water are sensitive to arrest by lack of calcium, lowered pH, or the presence of ether in their final medium. The time remaining until germinal vesicle breakdown is designated as "Stage II."

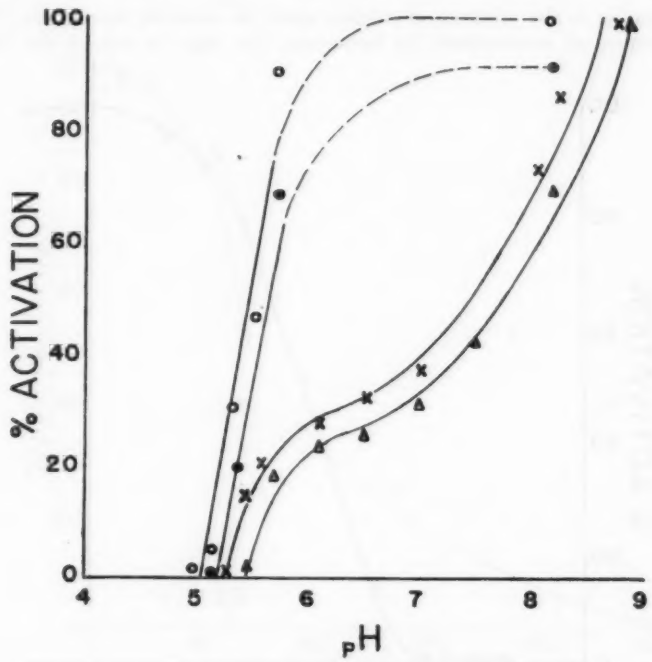


FIGURE 8. The influence of pH on artificial activation (pH adjusted by adding HCl to bicarbonate-free sea water). (o) represents 45 secs. ultraviolet irradiation; (●) represents 35 secs. irradiation; (×) represents hypotonicity, (△) represents hypertonicity. Dosages of stimulating agents arranged to give 90–100% activation at normal sea water pH.

D. Changes during Stage I

As mentioned earlier, slight changes of shape are often observed following sperm penetration or application of activating agents. Surface wrinkling or small indentations are often seen after application of some agents and deep indentations after others (*cf.* Tables I, II; Plate I). Artificial activating agents often evoke changes of shape more exaggerated than those resulting from fertilization. Eggs which are stimulated by ultraviolet light show marked wrinkling at the surface; sodium ions, isotonic urea (alone or in mixtures with sea water), heat and cold produce deep indentations which last from seconds to minutes. Such changes

in shape cannot be induced in the absence of calcium. Departure from spherical shape can be associated with changes in volume. In haematocrit experiments it was found that a decrease of at least 6–8% in volume follows stimulation by heat, urea, and sodium chloride if calcium is present. Decrease in volume means expulsion of water from the protoplasmic gel (syneresis). Such a gelation was detected by the centrifuge method after heat stimulation, which treatment also caused the

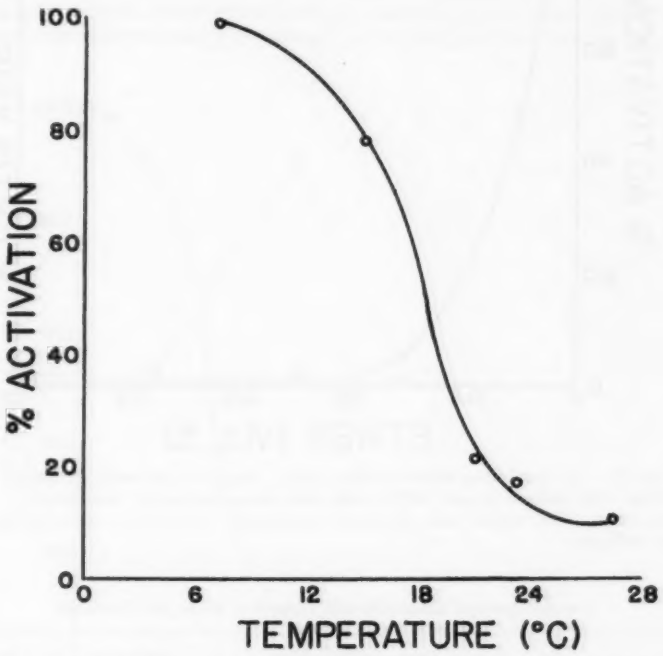


FIGURE 9. The effect of reduced temperature on excitability. The temperature of stimulation was 27° C. The dosage of ultraviolet light was chosen to give about 10% activation at this temperature. Aliquots of the stimulated eggs were transferred immediately to sea water at a lower temperature.

most prolonged indentations. The brevity of response to other agents made similar viscosity measurements by this method impossible. (Viscosity measurements are very difficult in unfertilized *Spisula* eggs because forces of the order of 200,000 times gravity are required to stratify the particulate matter in the cytoplasm.) There is a rapid increase in the rate of brownian movement at the end of Stage I; It is at about this time that the cell regains its spherical shape, probably through the uptake of water (imbibition).

The application of mixtures of two or three of the sea water cations in the proportions found in natural sea water shows some effects of ion antagonism on

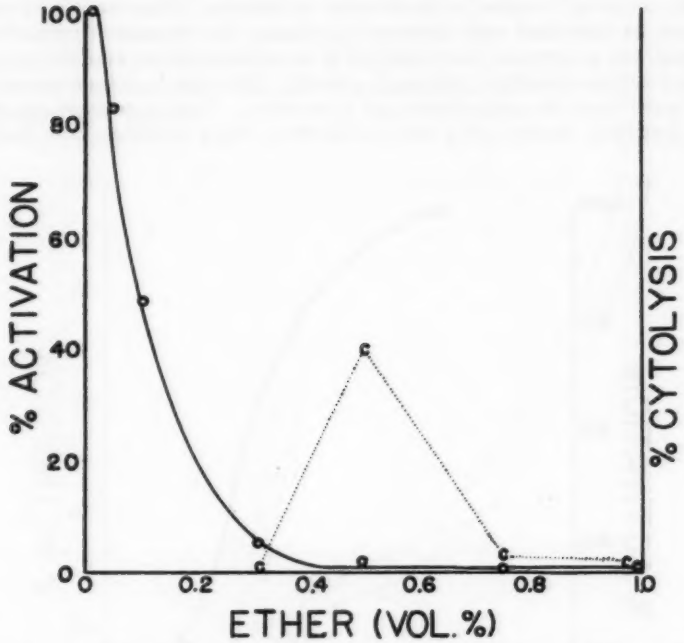


FIGURE 10. The inhibiting effect of ethyl ether. Eggs in sea water stimulated by dosage of ultraviolet light chosen to give 100% activation and subsequently transferred to sea water containing ether. The dotted line represents percentage of cytolysis (plotted on the same numerical ordinate).

TABLE II

Ion antagonism associated with changes of shape on stimulation*

Na^+	K^+	Ca^{++}	Mg^{++}	Wrinkling	Indentations
+	−	−	−	Marked	Few
+	+	−	−	Very slight	None
+	−	+	−	Marked	Marked
+	−	−	+	Slight	None
+	+	+	−	Few slight	None
+	+	−	+	None	None
+	−	+	+	Slight	None
+	+	+	+	None	None
−	+	+	−	None	None
−	+	+	+	Slight, prolonged	None
−	+	−	+	None	None
−	−	+	−	None	None

* Eggs were transferred with a minimum of sea water to mixtures of the isotonic cation chlorides. Thus in these mixtures, traces of all of the sea water cations were present. In the case of calcium, sufficient quantities of this ion were present to permit activation or changes of shape. The further addition of calcium intensified the shape changes.

changes of shape induced by sodium ions. Sodium ions in the presence of traces of calcium elicit sudden strong indentations. Table II shows that an excess of calcium intensifies the magnitude of the sodium response as long as the other cations are absent. Both potassium and magnesium alone weaken this response, and the two together abolish it. This is probably the reason that eggs do not normally show indentations when placed in sea water. It is true that freshly shed eggs are irregular in outline; this seems to be due to previous compression in the ovary. Hydrogen ions tend to prolong indentations caused by activating agents. Ether will not prevent indentations even though it inhibits germinal vesicle breakdown.

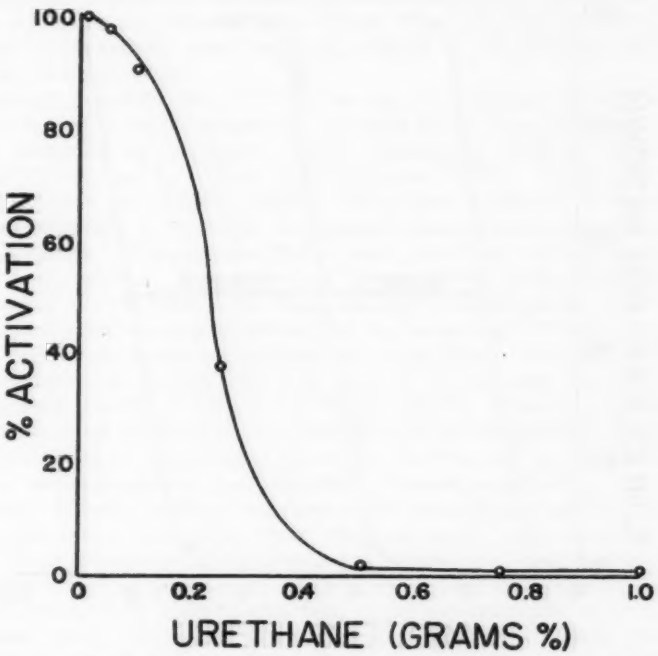


FIGURE 11. The inhibiting effect of ethyl urethane on stimulation by ultraviolet light. Dose of irradiation chosen to give 100% activation.

In fact, higher concentrations of ether can induce deep indentations. In living eggs, the removal of calcium by 10–20% sodium citrate in calcium-free sea water is the only way to prevent changes of shape following stimulation; this inhibition is reversible. Strong poisons may act similarly in preventing changes of shape, but their effects are not reversible. Thus iodoacetate (4×10^{-4} M but not 3×10^{-4} M) will inhibit changes of shape. Cyanide (10^{-3} M), or bromide (10^{-2} M) have no detectible inhibitory effect. Attempts at causing indentations with sodium ATP (up to 10^{-2} M) failed. A series of experiments was per-

formed in which eggs were transferred first to dilute sea water and then to various stimulating solutions of the same dilution (osmotic strength). Such a transfer of eggs from sea water to 50% sea water, and then to 50% isotonic urea resulted in surface indentations. Below this dilution, indentations were not detected. In another experiment indentations were observed following transfer from 35% sea water to 35% isotonic sodium chloride. In all of these experiments, and those summarized in Table II, the traces of calcium which accompanied the eggs when they were transferred from sea water to the test solutions were sufficient to permit changes of shape, and in some cases, nuclear breakdown.

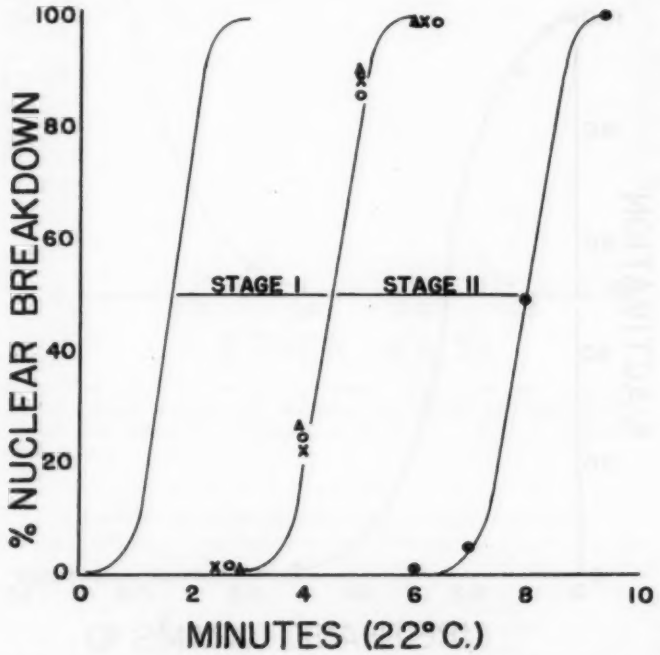


FIGURE 12. The duration of sensitivity to lack of calcium, acid sea water, or ether in eggs activated in normal sea water and subsequently transferred to these inhibitors. The right-hand curve represents the time at which dissolution of the germinal vesicle membranes is observed following fertilization. The left-hand curve is a hypothetical curve of the same slope, representing the probable penetration times of the sperm. The middle curve represents the time at which the fertilized eggs lose their sensitivity to inhibition by lack of calcium (o), acid sea water (×) and ether (▲). The time differences between the curves are called "Stage I" and "Stage II" as indicated in the figure.

During the course of Stage I there is measurable acid production in fertilized or artifically activated eggs. This is especially noticeable when eggs are suspended in unbuffered, bicarbonate-free sea water.

Discussion

A. Changes at the surface of the egg following activation

In the eggs of many marine invertebrates the penetration of the sperm causes striking changes at the cell surface. Although eggs of the sea urchin and other echinoderms exhibit highly elevated fertilization membranes, in general, the eggs of annelids and molluscs do not. There may be slight membrane elevation in these forms, however. Furthermore, in *Spisula* eggs, the cortical granules do not appear to be altered by fertilization or by artificial activation as they are in the sea urchin (Moser, 1939a, 1939b; Runnström, 1949), except perhaps under very severe conditions of artificial activation by ultraviolet light in synergism with an excess of one of the sea water cations. Under these conditions, widely elevated membranes are sometimes seen, and it is difficult to tell whether the cortical granules are changed or not.

The surface, especially the cortex of the egg, is important during the initial response of the egg to activating agents. Evidence for the importance of the cortex has been presented by Whitaker (1931), Runnström (1923), Monroy and Montalenti (1947) and by Wilson and Heilbrunn (1952). Smith and Clowes (1924), and Tyler and Schultz (1932) showed that a number of marine eggs could cleave normally at hydrogen ion concentrations greater than required to inhibit fertilization. This suggests that at least part of the fertilization reactions take place at the surface. Runnström and Kriszat (1952) believe that the cortex plays a "master role" in initiating a "chain reaction" at fertilization.

Some information concerning the role of the cortex can be inferred from the effects of various environmental changes on excitability. According to current opinion, the cortex is probably the only part of the cell into which ions from the environment can freely diffuse (*cf.* Mazia, 1940). Since minor changes in the ionic constitution of the sea water environment have an immediate and maximal effect on excitability, it seems logical to suppose that the part of the cell primarily involved in response reactions has come into a dynamic equilibrium with the new environment. It seems probable that these minor ionic changes must be acting primarily on the cortex to produce their effect so rapidly and drastically. Furthermore, minor changes in ionic environment which cause significant excitability changes have no detectible effect on cell division or later development. The fact that slight alterations in ionic balance tend to act synergistically with ultraviolet light to cause widely elevated membranes to appear indicates that these alterations do bring about cortical changes. That cell division proceeds in sea water modified in this way is evidence that the interior of the cell is not seriously affected. Thus the data on excitability changes under altered environmental conditions can be interpreted in terms of an effect on the cortex. These changes may well be related to the changes in cortical viscosity such as those described by Wilson and Heilbrunn (1952). However, other factors may be affected as well.

It is believed that, generally, the internal hydrogen ion concentration of a cell is maintained relatively constant over a wide range of external pH. Tyler and Schultz (1932) showed that fertilization was particularly sensitive to slightly acid sea water (pH 6.8–7.5). The excitability of *Spisula* eggs varied with external hydrogen ion concentration (Figs. 7 and 8). Accordingly, the curves of acid inhibition of activation could be interpreted as inactivation curves of some surface

enzymes, or processes occurring at the surface. Another possibility is that acid is affecting the dissociation of calcium from its protein binding, thereby disturbing the normal release of calcium following stimulation. Such a release of calcium could conceivably pass around the surface of the egg, as Moser (1939a, 1939b) believed, in a chain reaction similar to that postulated by Runnström and Kriszat (1952). However, the different curves of acid inhibition obtained with ultraviolet light on the one hand, and with osmotic stimulation on the other, might indicate somewhat different pathways for the response mechanism for these different stimulating agents, and for fertilization as well.

It has been shown (Fig. 2) that a lack of an excess of potassium in the normal environment can cause activation. Activation by potassium-free sea water is actually sodium activation. Although sodium and potassium ions both stimulate, they act antagonistically toward the cell. It may be primarily this antagonism which keeps the *Spisula* egg in the germinal vesicle stage until it is fertilized, but it is hard to imagine how the sperm or other stimuli could exert an effect by influencing this kind of a block, unless by a permeability change, for which there is at present no evidence in the *Spisula* egg. A similar situation of sodium-potassium antagonism appears to exist in the eggs of *Urechis* (Scheer and Scheer, 1947). Other factors, especially the divalent ions, pH and temperature doubtless also contribute to block activation. Wilson and Heilbrunn (1952) believe that either sodium or potassium can release calcium from its binding with protein in the cortex. Why these ions both stimulate (*cf.* results of Sawada, 1952, on *Mactra veneriformis*) and yet act antagonistically is not known. Sodium and potassium seem to act differently in their ability to initiate development (Allen, 1952b). The time required for nuclear breakdown is the same if eggs are activated by potassium (sea water with double potassium concentration) as if they are activated by sodium (potassium-free sea water). This indicates that developmental processes are initiated at the same time in both media. Eggs activated by sodium can be removed after a minute and developmental changes continue in sea water; eggs activated by potassium, however, must remain in the stimulating solution throughout Stage I (four to five minutes). If they are removed during this period and placed in sea water, the developmental processes already initiated will become reversed, as if they had been placed in an inhibitor.

Calcium plays an important role in the excitation of marine eggs and other cells. The necessity of calcium for fertilization and artificial activation is well known (see especially Loeb, 1915; Dalcq, 1928b; Pasteels, 1935). Of the parthenogenetic agents tried by Wison and Heilbrunn (1952), calcium was the only one which caused the cortex to stiffen. As a parthenogenetic agent, calcium is unusual in the length of exposure it requires to be effective. The results of this study (see Fig. 4) indicate that calcium enters the cortex and causes a decrease in excitability. Similarly, if calcium is withdrawn from the environment (and as a result, from the cell), excitability is increased. For many eggs, including *Spisula,* calcium is a poor activating agent. The parthenogenetic action of calcium in some eggs may be due to a leakage through the cortex; for, once within the cortex this calcium may imitate the effect of normal release of calcium by stimulating agents. The effects of calcium on excitability can be explained by postulating that agents which liquefy the cortex (*cf.* Wilson and Heilbrunn, 1952) act synergistically with one another and antagonistically with those agents, such as calcium,

which tend to stiffen the cortex. In this way, it would be possible to account for the action of the cations on excitability.

Magnesium acts as an anesthetic in *Spisula* eggs as in other cells (Wilbur, 1939; Sawada, 1952). Its effect on cortical viscosity has not been determined in marine eggs.

Ether is a good anesthetic for *Spisula* eggs and works best on activation when its concentration is below 0.3% (by volume). At slightly higher concentrations (about 0.5%) ether causes wide-spread cytolysis. At still higher concentrations this cytolysis is prevented, but the eggs will now cytolyze if returned to normal sea water. It is possible that ether causes cytolysis by some process which resembles stimulation, such as a release of calcium or an increase in permeability. The prevention of cytolysis at higher concentrations may be a direct inhibition of the enzymes responsible for disintegration of the cell.

The fact that activation in *Spisula* eggs is favored by reduced temperature (Fig. 9) does not support the theory of R. S. Lillie (1941 and earlier) for activation of starfish eggs. Much of the evidence gathered in this investigation is in opposition to Lillie's theory. Activation by some agents is so rapid in *Spisula* eggs that it is difficult to conceive of the formation of a complex activating substance such as Lillie postulated.

According to the colloidal theory of stimulation proposed by Heilbrunn and his students, all stimulating agents in general act on the same series of processes, namely, those which are involved in, or follow the release of calcium from protein binding in the cortex. Presumably the normal release of calcium would be brought about by a change in the binding capacity of the cortical proteins. An attempt was made to demonstrate that the binding capacity of these cortical proteins could be influenced by stimulating agents presented in the absence of calcium, with the result that subsequently added calcium would be able to initiate development. This was accomplished by ultraviolet stimulation (Fig. 5). Apparently the eggs recover from such ultraviolet effects, as evidenced by the decrease with time of their ability to respond to added calcium. Atttempts to duplicate these results with other activating agents failed.

The fact that egg excitability could be influenced after withdrawal of the stimulating agent, but only during the subsequent 4–5 minutes (Stage I), is apparently related to the fact that eggs are sensitive during this period to arrest by inhibitors (Fig. 12). Inhibition by acid at this stage suggests that a surface reaction is affected. Simultaneous susceptibility to lack of calcium suggests that this surface process requires calcium. It appears probable that the environmental changes and other agents which affect excitability do so by their influence on these surface processes occurring during Stage I. The presence of such an inhibitor-sensitive period following fertilization was first shown by Tyler and Schultz (1932). Similar experiments were performed by Goldstein (1953) on the activation (spontaneous maturation) of the *Chaetopterus* egg. Goldstein found two stages which were inhibited by lack of calcium and by carbon dioxide, respectively; however, this maturation process is not inhibited by acid sea water (above pH 3.5), whereas fertilization is inhibited at a much higher pH (7.1) in this egg (Smith and Clowes, 1924).

Although from much indirect evidence it would be tempting to believe that all

stimulating agents work by initiating the same sort of response reactions, the following evidence seems to suggest that there may be variations in pathway to different agents: (1) the pH-dependency curves for activation by different agents seem to be dissimilar (Figs. 7, 8); (2) sodium and potassium, which act antagonistically in sea water, each cause activation when presented in excess or not antagonized. Furthermore, those ions seem to influence the activation processes differently. (3) Activation can be obtained if eggs are irradiated by ultraviolet light in the absence of calcium and subsequently transferred to normal sea water. These results could not be duplicated with other activating agents.

II. Changes in the interior of the egg following activation

Following fertilization, chemical changes in the interior cytoplasm and nucleus prepare the egg for its metabolic needs during development (for discussion, see Runnström, 1949; Brachet, 1950). Colloidal changes in the cell interior probably also play a role in the processes leading to cleavage and further development (see Runnström, 1949; Heilbrunn, 1952). The fact that calcium is required for changes of shape (see Table II) caused by stimulation indicates that this ion is involved in these colloidal changes. For reasons mentioned earlier, it was not possible to obtain direct information concerning cytoplasmic viscosity changes at activation. The resistance of *Spisula* eggs to stratification by centrifugal force appears to be due mostly to their particularly high viscosity. However, this resistance to stratification might also be due in part to a lack of any marked difference in specific gravity between the granules and the ground substance. This possibility was indicated by the fact that the fat-containing granules collect somewhat more rapidly at the centripetal pole than do the heavier granules at the centrifugal pole when the eggs are subjected to forces of about 200,000 × gravity for from one to three minutes. The fact that the cytoplasm is a gel is also indicated by the formation of a ring of negative strain birefringence around the nucleus when this structure is moved through the cell by centrifugal force.

Changes in shape (indentations) similar to those found in the present investigation were described in *Spisula* eggs by Schechter (1941) in connection with natural changes which occur on aging. He showed that these changes were brought about more rapidly when calcium was present in the medium. It is thus interesting that calcium is associated with the occurrence of indentations as a result of both aging and stimulation.

It has been pointed out above that the changes of shape observed are actually a visible expression of syneresis (loss of water) by the egg protoplasm. Some similar changes in shape were reported by Tyler (1932) in the egg of the echiuroid worm, *Urechis caupo*. This egg already possesses a polar indentation before fertilization. The indentation disappears, reappears, and disappears for a second time before the germinal vesicle breaks down. Tyler made measurements to show that volume changes are involved, and that as the first indentation disappears, there is a drop in viscosity. Possibly the second indentation in *Urechis* is similar to the indentation observed in the present study. Tyler explained the changes in shape and volume by assuming changes in internal osmotic pressure. Since viscosity changes seem to have been involved as well, syneresis and imbibition may have been the immediate factors involved in the shape and volume changes. Presum-

ably, the forces developed by a contracting gel could oppose those of osmotic pressure. This certainly is true in the *Spisula* egg, as mentioned earlier in the results, where deep indentations occur even when the stimulating media are diluted with distilled water. Contractions or wrinkling at the surface of eggs seem to be of rather general occurrence (for example, see Runnström, 1949).

The loss of water by the egg is followed after a few minutes by a return of water (imbibition) and a rounding up of the egg's contour. This entrance of water causes a sharp increase in the rate of brownian movement, indicating liquefaction. It is at about this time that the processes designated as Stage I come to an end.

The rapidity with which eggs undergo cytolysis with disintegration following excessive stimulation is an indication of the relationship of stimulation to the activation of enzymes in the egg (for discussion of this point, see Runnström, 1949). Goldstein (1953) who has studied the maturation process in the eggs of the annelid worm, *Chaetopterus*, suggests that the nuclear membrane is dissolved by a calcium-activated proteolytic enzyme. There is ample evidence for the presence of proteolytic enzymes in the egg cell after fertilization (Lundblad, 1944; Woodward, 1950; Gross, 1952). It has been observed (Allen, 1951b) that in several marine eggs, the breakdown of the germinal vesicle is preceded by dissolution of the nucleolar membrane. It is thus possible that if an enzyme is involved in nuclear and nucleolar breakdown, it may come from within the nucleus. Since such an enzyme would presumably be calcium-activated (because some calcium is necessary for nuclear breakdown) it could, as far as is now known about calcium-activated enzymes, be either proteolytic, as Goldstein suggests, or lipolytic.

Summary

1. The present study is a preliminary survey of fertilization and artificial activation in the egg of the surf-clam, *Spisula solidissima* (Dillwyn).

2. The structure of the egg, optimal conditions for fertilization, and normal early development of fertilized and artificially activated eggs are described.

3. The results of treatment by various parthenogenetic agents are discussed with particular reference to possible similarities and differences in their mode of action and pattern of response initiated. Among the agents discussed are: ultraviolet irradiation, potassium, sodium, ammonia, osmotic stimuli, heat, cold, urea, and protamine (clupein).

4. The influence of various changes in environmental conditions has been investigated and the results correlated with the function of the cortical region of the cytoplasm during the first four or five minutes immediately following stimulation. Monovalent cations, temperature shock, and stimulation in the cold all increase excitability. Divalent ions, stimulation at slightly elevated temperatures, lowered pH, or the addition of ether, urethane or egg jelly decrease excitability.

5. Sodium-potassium antagonism may be largely responsible for maintenance of the egg in the germinal vesicle stage prior to fertilization.

6. The changes which can be detected in the interior cytoplasm and in the nucleus following activation are discussed with particular reference to changes of shape and volume, and to nuclear breakdown.

7. It is concluded that shape changes are probably caused by an expulsion of water (syneresis) from the cytoplasmic gel when the egg is activated. This expul-

sion of water is probably due to an increased gelation caused by a release of calcium from the cortex by activating agents.

8. The response mechanism of the egg can be divided into two stages on the basis of the fact that for 4–5 minutes after activation it is susceptible to inhibition by acid sea water, by lack of calcium, or by dilute ether; but after this time inhibition by these agents is no longer possible.

9. Evidence concerning the direct cause of germinal vesicle breakdown is discussed, and this evidence suggests that a calcium-activated proteolytic or lipolytic enzyme is involved.

LITERATURE CITED

ALLEN, R. D., 1951a. The use of *Spisula solidissima* eggs in cell research. *J. Cell. Comp. Physiol.*, **37**: 504–505.

ALLEN, R. D., 1951b. The role of the nucleolus in spindle formation. *Biol. Bull.*, **101**: 214.

ALLEN, R. D., 1951c. Antimitotic substances secreted from eggs. *Biol. Bull.*, **101**: 214.

ALLEN, R. D., 1952a. A visible response to stimulation in *Spisula* eggs. *Biol. Bull.*, **103**: 283.

ALLEN, R. D., 1952b. The behavior of *Spisula* eggs with respect to potassium ions. *Biol. Bull.*, **103**: 290–291.

BRACHET, J., 1950. Chemical embryology. Interscience Press, New York.

DALCQ, A., 1928a. Les bases physiologiques de la fécondation et de la parthenogénèse. Presses Universitaires, Paris.

DALCQ, A., 1928b. Le rôle du calcium et du potassium dans l'entrée en maturation de l'oeuf de *Pholade* (*Barnea candida*). *Protoplasma*, **4**: 18–44.

GOLDSTEIN, L., 1953. A study of the mechanism of activation and nuclear breakdown in the *Chaetopterus* egg. *Biol. Bull.*, **105**: 87–102.

GROSS, P. R., 1952. A study of colloidal changes in sea urchin egg homogenates. *Biol. Bull.*, **103**: 293.

HEILBRUNN, L. V., 1952. An outline of general physiology. W. B. Saunders Co., Phila. Third Edition.

KOSTANECKI, K., 1908. Zur Morphologie der künstlichen parthenogenetischen Entwicklung bei *Mactra*. *Arch. f. Mikr. Anat.*, **72**: 327–352.

LILLIE, R. S., 1941. Further experiments on artificial parthenogenesis on starfish eggs, with a review. *Physiol. Zool.*, **14**: 239–267.

LOEB, J., 1913. Artificial parthenogenesis and fertilization. Chicago Univ. Press, Chicago.

LOEB, J., 1915. On the nature of the conditions which determine or prevent the entrance of the spermatozoan into the egg. *Amer. Nat.*, **49**: 257–285.

LOOSANOFF, V. L., AND H. C. DAVIS, 1950. Conditioning *V. mercenaria* for spawning in winter and breeding its larvae in the laboratory. *Biol. Bull.*, **98**: 60–65.

LUNDBLAD, G., 1944. Proteolytic activity in eggs and sperms. *Nature*, **163**: 643.

LYMAN, J., AND R. H. FLEMING, 1940. Composition of sea water. *J. Mar. Res.*, **3**: 134–146.

MAZIA, D., 1940. The binding of ions by the cell surface. *Cold Spring Harbor Symp. Quant. Biol.*, **8**: 195–203.

MONROY, A., AND G. MONTALENTI, 1947. Variations of the submicroscopic structure of the cortical layer of fertilized and parthenogenetic sea urchin eggs. *Biol. Bull.*, **92**: 151–161.

MORRIS, M., 1917. A cytological study of artificial parthenogenesis in *Cumingia*. *J. Exp. Zool.*, **22**: 1–51.

MOSER, F., 1939a. Studies on a cortical layer response to stimulating agents in the *Arbacia* egg. I. Response to insemination. *J. Exp. Zool.*, **80**: 423–445.

MOSER, F., 1939b. Studies on a cortical layer response to stimulating agents in the *Arbacia* egg. II. Response to chemical and physical agents. *J. Exp. Zool.*, **80**: 447–471.

PASTEELS, J. J., 1935. Recherches sur le déterminisme de l'entrée en maturation de l'oeuf chez divers invertebrés marins. *Arch. de Biol.*, **46**: 229–262.

RUNNSTRÖM, J., 1923. Eine lipoide Oberflächenschicht bei dem Seeigelei. *Acta Zool.*, **4**: 285–311.

RUNNSTRÖM, J., 1949. The mechanism of fertilization in metazoa. *Advances in Enzymology*, **9**: 241–327.

RUNNSTRÖM, J., AND G. KRISZAT, 1952. The cortical propagation of the activation impulse in the sea urchin egg. *Exp. Cell. Res.*, **3**: 419–426.

SAWADA, N., 1952. Effect of some cations on the breakdown of the germinal vesicle in the egg of the clam, *Mactra veneriformis*. *Bull. Exp. Biol.* (Japanese), **2**: 9.

SCHECHTER, V., 1941. Experimental studies upon the egg cells of the clam, *Mactra solidissima*, with special reference to longevity. *J. Exp. Zool.*, **86**: 461–479.

SCHEER, B. T., AND M. A. R. SCHEER, 1947. Some interrelations of drug and ion actions in the artificial activation of marine eggs. *Physiol. Zool.*, **20**: 15–32.

SCOTT, G. T., AND H. R. HAYWARD, 1950. The influence of the glycolytic inhibitor iodoacetic acid on aging and on the potassium and sodium content of the egg cells of *Mactra solidissima*. *Biol. Bull.*, **99**: 363–364.

SMITH, H., AND G. H. A. CLOWES, 1924. The influence of hydrogen ion concentration on the fertilization process in *Arbacia*, *Asterias* and *Chaetopterus* eggs. *Biol. Bull.*, **47**: 333–344.

TYLER, A., 1932. Change in volume and surface of *Urechis* eggs upon fertilization. *J. Exp. Zool.*, **63**: 155–173.

TYLER, A., 1941. Artificial parthenogenesis. *Biol. Revs.*, **16**: 291–336.

TYLER, A., AND J. SCHULTZ, 1932. Inhibition and reversal of fertilization in eggs of the echiuroid worm, *Urechis caupo*. *J. Exp. Zool.*, **63**: 509–531.

WHITAKER, D. M., 1931. On the conduction of the cortical change at fertilization in the starfish egg. *Biol. Bull.*, **60**: 23–29.

WILBUR, K. M., 1939. The relation of the magnesium ion to ultraviolet stimulation in the *Nereis* egg. *Physiol. Zool.*, **12**: 102–109.

WILSON, W. L., AND L. V. HEILBRUNN, 1952. The protoplasmic cortex in relation to stimulation. *Biol. Bull.*, **103**: 139–144.

WOODWARD, A. A., 1950. Proteolytic enzymes in the eggs of the clam, *Mactra solidisssima*. *Biol. Bull.*, **99**: 367.

TISSUE RESPIRATION, GROWTH, AND BASAL METABOLISM

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It is a phenomenon general in mammals and other classes of the animal kingdom that metabolic rate per unit weight decreases with increasing body size. This is expressed in the surface rule of Rubner who stated that metabolic rate decreases per unit weight, but is constant per unit surface. More recent investigations (Brody, 1945; Kleiber, 1947) indicate that interspecifically, *i.e.*, comparing mature animals of different species, basal metabolic rate in mammals is proportional rather to a $\frac{3}{4}$ power of weight than to surface or the $\frac{2}{3}$ power of weight. Intraspecifically, *i.e.*, comparing animals of different body size within the same species, the surface rule applies to the general trend of the size-metabolism relation in rats, although qualifications have to be made in detail (Bertalanffy, Müller and Racine, unpublished data). These complications, however, do not alter the fundamental fact of the decrease in weight-specific metabolic rate with increasing body size. However, we do not have a satisfactory explanation for this phenomenon.

The basic alternative seems to be whether the dependence of metabolism on body size is based upon *cellular* or *organismic* factors. It may be due to intrinsic differences in the metabolism of the cells of smaller and larger individuals which will show up also in isolated tissues; or it may be due to regulative factors lying in the organism as a whole. There may be also a combination of both.

Earlier work on the relation between tissue metabolism and body size (reviewed by Kleiber, 1947) is contradictory. Terroine and Roche (1925), and, independently, Grafe (1925; Grafe, Reinwein and Singer, 1925), stated that the metabolic rate per unit weight of homologous tissues *in vitro* is essentially the same for small and large animals, although basal metabolic rate per unit weight *in vivo* decreases systematically with increasing body size. Grafe assumed that the metabolic rate of tissue *in situ* is checked by central regulators, mainly the nervous and endocrine system. On the other hand, LeBreton and Kayser (1926; Kayser, LeBreton and Schaeffer, 1925), and Borger and Groll (1926) reported variation of the respiration rate of tissues with increasing body size, in individuals of the same species as well as in different species. This earlier work is open to criticism, and Grafe *et al.*'s calculations, in particular, were based upon erroneous assumptions (*cf.* Field *et al.*, 1939).

More recent results, however, are also contradictory. According to Field *et al.* (1939) the summated tissue respiration (*i.e.*, metabolic rate *in vitro* per unit fresh weight, multiplied by the weight of the respective organ, and summated over 20 main organs) amounts to 66% of the basal metabolic rate of the rat, and, if allowance is made for minimal functional activity (muscle tone, cardiac, respiratory, smooth muscle, secretory function), even for 89% of the respiration of the intact animal. The authors conclude that basal metabolism is the arithmetic sum

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of tissue respiration as measured by the Warburg technique, and that there is no reason to assume that the level of tissue respiration *in situ* is determined by organismic factors which are not operative *in vitro*. Similar experiments were made by Martin and Fuhrmann (1941) on the dog, where summated tissue respiration gives 79% of the resting metabolism. It is concluded, therefore, that resting metabolism can be accounted for by summated tissue respiration and minimal functional activity.

However, it is well-known that the respiration rates of tissues show considerable variation, depending on the medium used. In serum, or in media containing the relevant metabolites (Krebs, 1950), they are often two or three times the values obtained in saline. It appears, therefore, that it is hard to decide which values should be considered to be the "true" metabolic rates of tissues corresponding to those *in situ*, so that these values, multiplied by organ weight, would give the total respiration of the organ in question. If, instead of saline, the previously mentioned authors had used serum, they would probably have obtained not 66%, but more than 100% of basal metabolic rate as summated tissue respiration.

Kleiber (1941; Weymouth, Field and Kleiber, 1942) investigated tissue respiration of liver from rats, rabbits, sheep, horse and cow, and found that Qo_2 decreases to the same extent (namely, the $-\frac{1}{4}$ power) as does the basal metabolic rate per unit weight, according to the $\frac{3}{4}$ power rule. He concludes (1941; p. 422), therefore, that "the factors which determine the metabolic level *in vivo* seem still to be present in the surviving tissue cut out of the organism." Similarly, Weymouth *et al.* (1944) studied the Qo_2 of the midgut gland of individuals of different size in the kelp crab *Pugettia*. They also found a systematic decrease of the Qo_2 values with increasing size, paralleling the decrease in weight-specific basal metabolic rates of the intact animals. Krebs (1950) compared the Qo_2 values of 5 tissues of 9 mammalian species. He found that, in general, the Qo_2 values of the larger species are somewhat lower than the homologous values of small species; but there is no parallel decrease in the different tissues, nor a consistent relation to the decrease of weight-specific basal metabolic rate with increasing size. The greatest decrease of Qo_2 in species of larger size is found in the liver (comparable to Kleiber's results though with some exceptions); but in the other tissues the decrease in Qo_2 is much smaller than the decrease in basal metabolism.

There seems to be, as yet, no systematic investigation on the intraspecific size dependence of the Qo_2 of different tissues. The present work (*cf.* Bertalanffy and Pirozynski, 1951; Pirozynski and Bertalanffy, 1952) was started before Krebs' investigation came to our attention.

The preliminary report of our results (Bertalanffy and Pirozynski, 1951) has been followed by an interesting investigation of tissue respiration of kidney and liver in growing chicken (Crandall and Smith, 1952) which confirms the results and conclusions of the present work. Three groups of chickens (body weight 66, 178, and 2350 gm.) were investigated. Apart from an early minimum of total metabolism as well as of liver Qo_2 after hatching, characteristic of chicken and not present in the rat, the relations found correspond to our results: No correlation between body size and Qo_2 in the kidney, a slight decrease of Qo_2 ($a \sim 0.1$) with increasing body size in liver slices.

A remark seems to be appropriate as to the relative value of interspecific and intraspecific comparisons. The first has, of course, the advantage of allowing a much greater range of body sizes to be compared; further, adult animals are compared so that developmental differences cancel out. On the other hand, although the physiological differences between newborn and adults are great, the same is true for the anatomical, physiological, biochemical, ecological, etc. differences even between related species, not to speak of comparisons "from the mouse to the elephant." The startling fact is that in spite of this, simple quantitative relations in basal metabolism can be established, intraspecifically as well as interspecifically.

Material and Methods

In our experiments male and female albino rats (Wistar strain) were used, representing a continuous series from newborn animals of 9 gm. body weight to adults of 392 gm. The animals, except for the newborn, were kept for 24 hours in individual cages prior to the experiment. All rats were fed Purina Fox Chow and tap water *ad libitum*, before the actual experiment took place. The diet was restricted to tap water only for 12 to 18 hours (basal metabolism regime). All animals were killed by breaking the cervical vertebra.

The organs to be investigated were carefully removed immediately after death and placed in ice-cold saline. The pieces of whole organs were sliced by free hand according to the method introduced by Deutsch and Raper (1936). Tissues were sliced up to the thickness of 0.4 mm. except the diaphragm, which was wholly removed from the animal by means of sharp scissors or a razor blade, and cut into two or three pieces. The muscular parts were then carefully separated from the adjacent connective tissue and placed in the flasks. Before transferring the slices to the Warburg flasks, they were gently dried by touching with a piece of hard filter paper. The slices of organs from newborn rats were generally prepared in the same manner except the brain, which was cut in half and minced by means of a forceps before being placed in the flask. A few embryonic tissues were obtained from pregnant rats. The smaller embryos of 25–35 mgm. body weight were transferred into the flask after removing the fetal membranes; the larger specimens were sliced using the same technique as with the organs of adult animals.

The oxygen consumption was determined by the direct method of Warburg using the technique described by Umbreit *et al.* (1949). The oxygen uptake in Krebs-Ringer-phosphate solution of pH 7.4 was measured in pure oxygen with carbon dioxide absorbed by alkali-soaked filter paper in the center well. The flasks attached to the manometers were placed in a constant temperature bath of 37.0° C. ($\pm$ 0.1) and shaken at the rate of 124 oscillations per minute. The equilibration period in the water bath was approximately 20 minutes. The standard experimental period was 60 minutes with readings taken every 10 minutes. The dry weight of the investigated tissue was determined on an analytical balance after drying the slices for at least two hours at 105–110° C.

Higher Q_{O_2} values can be obtained in other media. However, in phosphate Ringer we found the Q_{O_2} values to remain approximately constant during the experimental period, except in the case of brain (Fig. 1), while if glucose or other metabolites are added, the Q_{O_2} values often decrease. Since our study amounts

to a comparison under standard conditions, it was preferable to use a medium where there is no decrease of Q_{O_2} during the experiment, and therefore no need to make extrapolations.

The order of the differences to be expected if the decrease of basal metabolic rate is based upon differences in tissue respiration can be estimated as follows. Basal metabolism is generally proportional to a power of the weight:

$$M = bW^{\alpha}, \tag{1}$$

where M is the rate of basal metabolism, W the body weight, the exponent α indicates the slope of the regression line in log-log plot, and b is a constant, indicating the extrapolated value of M for $W = 1$. The dependence of metabolism on body size is a special case of the general law of allometric growth (*cf.* Bertalanffy,

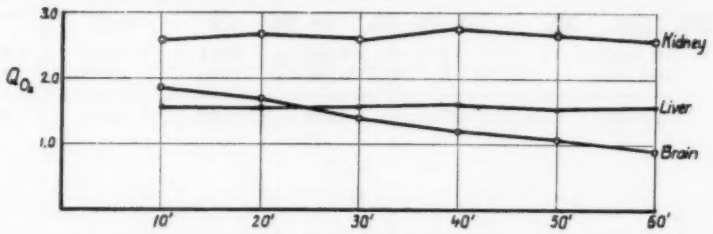

FIGURE 1. Q_{O_2} as a function of time. Typical experiments.

1951a). In the case of the surface rule, $\alpha = \frac{2}{3}$. The surface rule holds, although with qualifications, for intraspecific comparison in the rat (Bertalanffy, Mueller and Racine, unpublished); this is also stated by Kleiber (1947), according to Benedict's data (1938). Therefore,

$$M/W = bW^{-1/3} \tag{2}$$

applies for metabolic rate per unit weight. Thus, the exponent α should be $-.33$ in the case of the surface rule (or $-.25$ for the $\frac{3}{4}$ power rule). If, for example, the weights compared are $1:2:4:8:16$, metabolic rates per unit weight (a measure of which is Q_{O_2}) should decrease in the ratio $1:.79:.63:.50:.4$, in the case of the surface rule. Differences of this order should be readily detected by the Warburg method.

The statistical evaluation of the data, and calculation of α, b, $S_{(\log y \cdot \log x)}$ (standard error), and ρ (coefficient of correlation) were made according to the method indicated by Brody (1945, pp. 398 ff.). In Figures 2–8, the central line gives the regression of Q_{O_2}, and the two parallel lines give the standard error in per cent, including $\frac{2}{3}$ of the cases.

RESULTS

The results are indicated in Table I and Figures 2–8. As to the individual organs, the following remarks can be made.

TABLE I

Statistical evaluation of the relation of Qo_2 to body size in organs of the rat

Organ	N	a	b	$S_{(\log y \cdot \log x)}$	ρ
Heart	27	−0.050	10.83	0.102	0.229
Lungs	30	−0.085	10.33	0.073	0.501
Liver					
1st cycle:	30	−0.116	13.65	0.063	0.497
2nd cycle:	15	−0.018	8.59	0.074	0.107
Brain cortex	30	+0.047	6.89	0.064	0.263
Kidney cortex	43	+0.030	13.91	0.060	0.170
Thymus					
1st cycle:	11	−0.263	20.11	0.032	0.740
2nd cycle:	17	−0.253	29.46	0.068	0.514
Diaphragm	27	−0.258	18.92	0.066	0.929

Heart

The Qo_2 values are presented in Figure 2. There is a slight decrease with increasing body size, but the correlation coefficient is low, as can be seen from Table I.

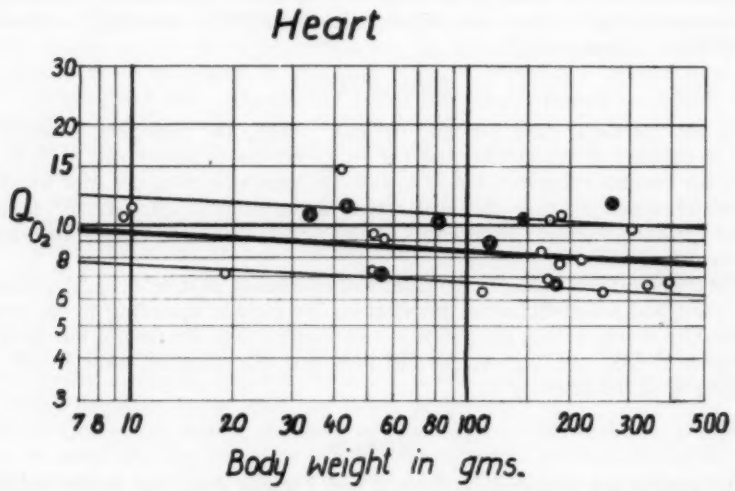

FIGURE 2. Qo_2 of heart in relation to body size. ○ = males, ● = females in Figures 2–8.

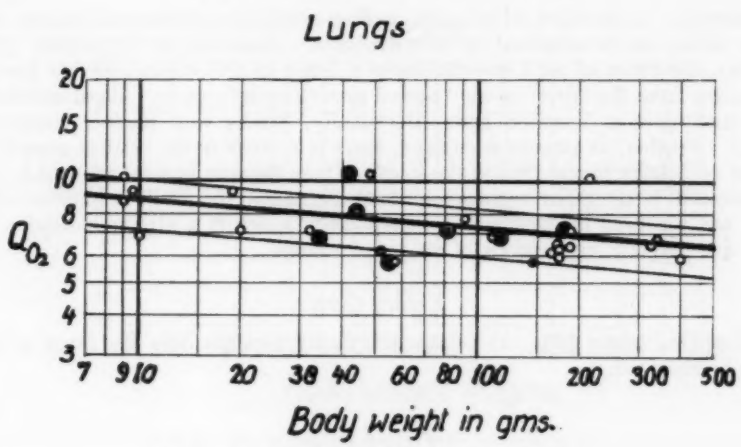

FIGURE 3. Q_{O_2} of lung in relation to body size.

Lung

The situation for the Q_{O_2} of lungs is similar to that of heart, as shown in Figure 3.

Liver

It appears that a break can be assumed in the allometric line of the Q_{O_2}, somewhere in the region of 100 gms. body weight (Fig. 4). This break would correspond to a break in the curve of the relative growth of this organ (Bertalanffy and Pirozynski, 1952). It seems that at this body weight and in this time which

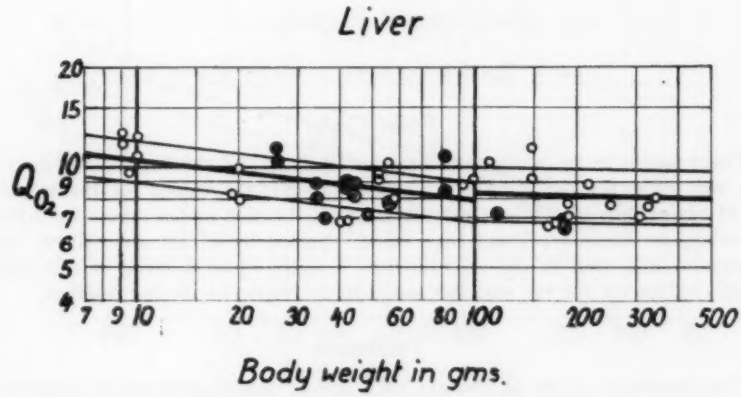

FIGURE 4. Q_{O_2} of liver in relation to body size.

corresponds to the start of puberty, a deep-reaching physiological change takes place which can be observed in different ways. According to Bertalanffy (1938, 1951a), the curve of total growth shows a break in this region, so that here the transition from the "first" to the "second growth cycle" occurs. Basal metabolism also undergoes a deviation here (Bertalanffy, Müller and Racine, unpublished data). Further, as already mentioned, there is a break in the relative growth and in the regression line of Qo_2 of the liver. These changes in total growth, in basal metabolism, in the relative growth of the liver, and in the tissue respiration of the liver are observed independently of each other which is a strong indication that they are different expressions of an actual change.

Kidney Cortex

The Qo_2 values (Fig. 5) remain practically constant over the range of body sizes investigated.

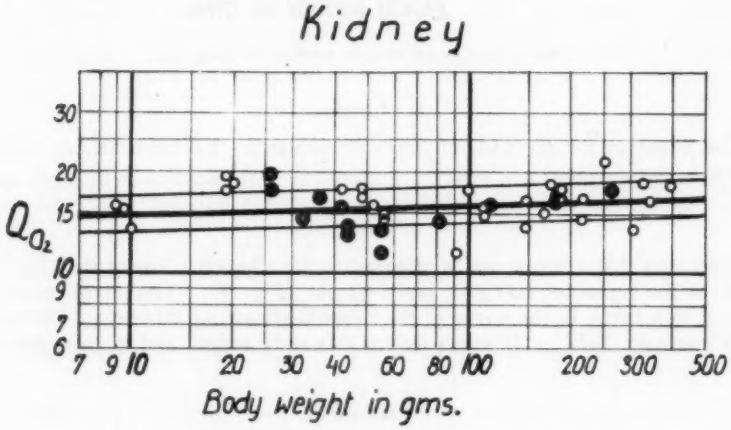

FIGURE 5. Qo_2 of kidney cortex in relation to body size.

Brain Cortex

There seems to be a slight increase of the Qo_2 of the brain with increasing body size (Fig. 6). According to Elliott (1948), the values for the respiration rate of brain show no obvious correlation with the size of the animals. Although our values for brain are lower than those reported by Elliott and others, because no glucose was used in our experiments, a slight upward trend is noticeable in Elliott's values for the rat, and this seems to correspond with our findings.

Diaphragm

The diaphragm is the only organ used in our experiments which shows a definite correlation of Qo_2 with body size, giving an exponent $\alpha = -.26$, and a high

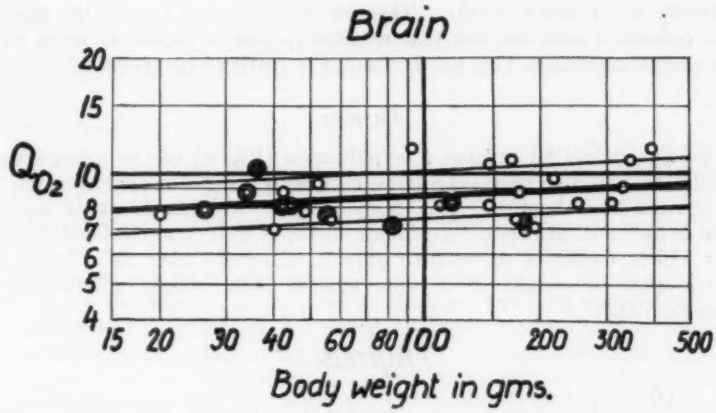

FIGURE 6. Q_{O_2} of brain cortex in relation to body size.

correlation coefficient, $\rho = .93$ (Fig. 7). It is to be noted that the diaphragm was not sliced as were the other tissues.

The marked decline of Q_{O_2} of the diaphragm with increasing body size may be connected with the continuous activity of this organ in respiration, since it serves oxygen uptake which, in total metabolic rate, shows a similar dependence on body weight. Also in interspecific comparison of adult rats and mice, there is a similar correlation of the Q_{O_2} of diaphragm to body size as is found intraspecifically

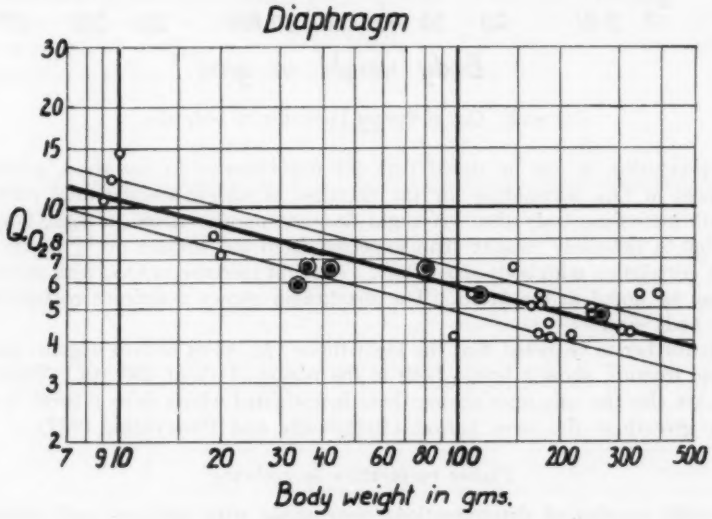

FIGURE 7. Q_{O_2} of diaphragm in relation to body size.

(Bertalanffy and Estwick, 1953). The size dependence of Q_{O_2} of the diaphragm may be connected with the fact that respiratory rate is higher in small as compared to larger animals. This will be studied in further experiments.

Thymus

In the Q_{O_2} values for thymus, a periodization (Fig. 8) can be found since they can be divided into two regression lines, each with a slope of approximately $-\frac{1}{4}$, and interrupted at about 100 gms. body weight. The break would again correspond to the "critical period" mentioned above, as well as to the involution of the thymus which, measured as relative growth, manifests itself as a break in the allometric plot of weight of thymus against body weight (Bertalanffy and Pirozynski, 1952).

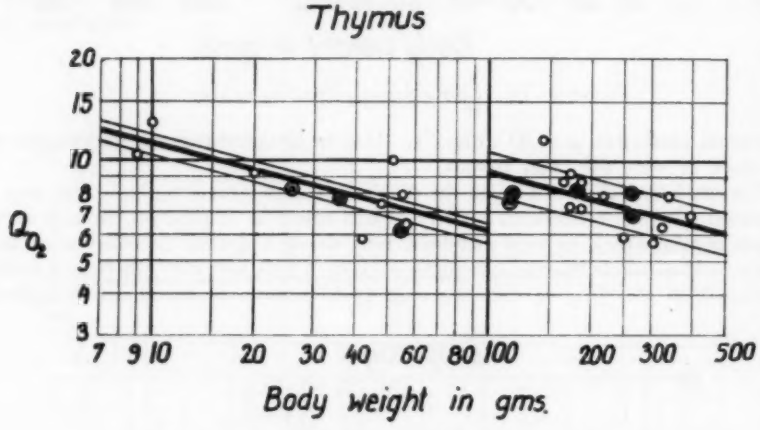

FIGURE 8. Q_{O_2} of thymus in relation to body size.

In conclusion, it can be stated that our experiments do not show systematic differences of Q_{O_2} accounting for the decrease of weight-specific total metabolic rate with increasing body size. A slight decrease may be noted in liver, lung and heart, but is definitely smaller than expected from the surface or $\frac{3}{4}$ power rule, and the correlation with body size is low. A slight increase in Q_{O_2} with increasing size may be noted in the brain. The diaphragm shows a definite correlation of Q_{O_2} to body size.

It is further to be noted that the logarithmic Q_{O_2} plots of two organs, namely, liver and thymus, show a break, both in the region of about 100 gm. These two organs are also the only ones among those investigated which show a break in their relative growth at the same period (Bertalanffy and Pirozynski, 1952).

Tissue respiration in embryos

A small number of determinations were made with embryos and embryonic tissues. Our values for naked fetuses are given in Table II. These results con-

TABLE II

Qo_2 of rat fetuses of different size

Body weight in mgm.	24.1	27.2	33.0	650.0
Qo_2	−6.24	−7.38	−7.82	−6.12

firm those of Kleiber, Cole and Smith (1943) who give for rat fetuses (naked) a mean Qo_2 of -7.2, with which our values are in good agreement. These authors estimate that the rate of oxygen consumption of the 13-day old rat fetus *in vitro* is only $\frac{1}{10}$ of the rate to be expected if the fetus behaved metabolically like a small independent homeotherm according to the $\frac{3}{4}$-power rule, or $\frac{1}{20}$ of the rate to be expected according to the surface rule. The fetal metabolic rate per unit moist weight is of the same order of magnitude as that of adult rats and considerably smaller than that of newly born to 12-day old rats. A few determinations of fetal organs are given in Table III. It appears that the Qo_2 of fetal liver is characteristically smaller than that of newborns.

DISCUSSION

1. *Interspecific and intraspecific comparison*

In order to compare intraspecific and interspecific size dependence of tissue metabolism, the data of Krebs (1950) were given a statistical treatment identical to that used for our own experiments. Krebs gives the Qo_2 for lung, liver, kidney cortex, brain cortex, and spleen in 9 species of mammals, ranging from the mouse to the horse, as found in the improved media introduced in his paper. The mean values of Qo_2, as given by Krebs (p. 259, Table IV) were calculated. A comparison of Tables IV and I shows the following facts.

TABLE III

Qo_2 of some fetal organs

Weight of fetus in grams	Brain cortex	Kidney cortex	Liver
0.65	−6.99		−7.45
	−7.52		−9.18
			−8.8
2.9	−8.08	−15.24	−7.13
			−9.38
			−8.83
			−8.14

1) The decrease in Qo_2, as measured by the exponent α, is, in all tissues studied by Krebs, considerably smaller than would correspond to the ¾-power rule (in the order of $\alpha = -.06$ to $-.14$, as compared to $-.25$, demanded by this rule). Therefore, interspecific comparison shows in the same way as does intraspecific comparison, that variations in tissue respiration, measured by the Warburg technique, cannot be the decisive factor for the decrease of weight-specific basal metabolic rate. Insofar as the brain is concerned, Krebs' determinations correspond well with those given by Elliott (1948). Elliott gives respiration per unit fresh weight in mice, rats, guinea pigs, rabbits, dogs, man and beef. He finds that respiration of cortex slices decreases inversely with body size, the exponent α being $-.1$. Since Elliott's regression line is drawn by estimate and without statistical calculation, this value is practically identical with the value obtained by statistical evaluation of Krebs' data ($-.07$).

2) The interspecific and intraspecific α-values are partly similar (liver, lung), partly they are significantly different (brain, kidney). This is an expression of a fact that is found also in morphological phenomena (Bertalanffy and Pirozynski, 1952), namely, that intraspecific and interspecific allometry (a special case of which are the relations between metabolic phenomena and body size) need not necessarily be identical.

TABLE IV

Statistical evaluation of Krebs' data on the relation of Qo_2 to body size in nine mammalian species

Organ	a	b	$S_{(\log y \cdot \log x)}$	ρ
Brain cortex	−0.069	35.69	0.029	0.969
Kidney cortex	−0.064	43.79	0.057	0.882
Liver	−0.115	23.43	0.063	0.949
Spleen	−0.139	20.21	0.059	0.968
Lung	−0.095	11.18	0.098	0.846

3) The values of the integration constant b, which indicates the extrapolated value of Qo_2 for a weight $W = 1$ gm., are much higher in Krebs' than in our data. This is a consequence of the fact that the media applied by Krebs give higher absolute Qo_2 values than those obtained in Ringer-phosphate, as used in our experiments.

4) The values of the standard errors $S_{(\log y \cdot \log x)}$, as given for our own and Krebs' data, are not directly comparable. Table I gives the standard errors, calculated for our individual determinations, while Table IV is calculated, as a matter of convenience, for the mean values obtained from a larger number of determinations. Nevertheless, the comparison is rather favorable for our results because the standard errors for our individual determinations are not larger than those for the average values given by Krebs.

5) The correlation between body size and tissue metabolism, as expressed by ρ, is much higher in interspecific than in intraspecific comparison. In other words, the factor of body size plays a greater role in determining the rate of tissue metabolism within different species of different size, than it does in determining tissue metabolism of individuals of different size within the same species.

2. The explanation of the relation between metabolism and body size

Our results lead to a number of conclusions with respect to possible explanations of the rule that weight-specific metabolic rate decreases with increasing body size.

1) A first explanation is that this decrease, as expressed in the surface or ¾-power rule, is due to intracellular factors, *i.e.*, to a corresponding decrease of the Qo_2 of the tissues the sum of which is the organism. Krüger (1940) suggested that the surface rule is based upon the principle of chemical allometry (Needham, 1934), and possibly upon the decrease in concentration of respiratory enzymes with increasing body size. Such decrease is actually found with respect to some systems taking part in respiration. According to Rosenthal and Drabkin (1943), the cytochrome *c* concentration decreases in the series mouse, rat, rabbit, dog, pig, man, and horse, with the −.278 power of body weight, *i.e.*, approximately according to the ¾-power rule. A decrease in glutathione with increasing body size was found by Gregory and Goss (1933) and Patrušev (1937). A similar concept was advanced by Weymouth *et al.* (1944). The coincidence of the values for total and liver respiration in rats, rabbits, and sheep, and equally for total respiration (interspecific and intraspecific comparison) and respiration of the midgut gland in crustaceans, leads these authors to the conclusion that "the regressions of the weight-specific rates for the different tissues apparently form, in the log-log plot, a family of parallel lines, some high and some lower, corresponding to the intensity of respiration, but all showing the same slope as the regression of the weight-specific rate of the entire animal" (p. 68).

There are two objections to be made against this argument. As far as crustaceans are concerned, total metabolism in *Pugettia* follows, according to Weymouth *et al.* (1944), the ¾-power rule. Investigations with other crustaceans (isopods: Müller, 1943b; Bertalanffy, 1951b; *Daphnia:* Jančařik, 1948; *Artemia:* Bertalanffy and Krywienczyk, 1953) show, however, that here the surface rule applies.

With respect to the general viewpoint, our results show that the extrapolation to all organs from one organ investigated—the liver in the rat, the midgut gland in crabs—is not justified. Our results, obtained in intraspecific comparison, correspond in this respect with the interspecific comparison, as carried through by Krebs: The regressions of Qo_2 with respect to body size are different for the various organs.

2) Krebs (1950), not finding a systematic decrease of the Qo_2 values investigated which would correspond to the decrease of weight-specific total metabolic rate, concludes (p. 266) that "the characteristic differences in the basal rate of heat production in animals of different size are to be attributed mainly to variations in the Qo_2 of the musculature." Krebs did not investigate the Qo_2 of muscles in animals of different size. His conclusion is based upon the fact that the muscles play a leading part in thermoregulation which, according to the classical explanation given by Rubner, is at the basis of the surface law. Krebs offers this explanation for interspecific comparison of metabolic rates in the series of mammals, but naturally it could be applied also to intraspecific comparison.

Two viewpoints are to be distinguished in this hypothesis. *a*) Insofar as muscular activity is a means of thermoregulation, this cannot be the basic principle in the decline of weight-specific metabolic rate with increasing body size. For the

latter is a phenomenon in no way specific of, or limited to homeotherms, but universal in most animal phyla (Bertalanffy, 1951b). The surface rule applies, even more unequivocally than in mammals, to cold-blooded vertebrates and many invertebrate classes. It was found to apply in roundworms (*Ascaris:* Krüger, 1940); in certain molluscs (*Lamellibranchiata:* Weinland, 1919; Ludwig and Krywienczyk, 1950; *Prosobranchia:* Krywienczyk, 1952a); in crustaceans (*Branchiopoda:* Jančařik, 1948; Bertalanffy and Krywienczyk, 1953; *Isopoda:* Müller, 1943b; Will, 1952); in fish (Bertalanffy and Müller, 1943) and reptils (Kramer, 1934). A decline of metabolic rates corresponding to the ¾-power rule is found in turbellarians (Bertalanffy and Müller, 1943) and in pond snails (*Limnaeidae:* Bertalanffy and Müller, 1943; Füsser and Krüger, 1951; Krywienczyk, 1952b). The only groups where no decline of weight-specific metabolic rates is found, *i.e.,* where total metabolism is directly proportional to weight, are land snails (*Helicidae:* Liebsch, 1929; Bertalanffy and Müller, 1943) and insects (Kittel, 1941; Bertalanffy and Müller, 1943; Müller, 1943a; Will, 1952)—possible due to pecularities of their respiratory mechanisms. Thus, homeothermy and muscular activity in its service is certainly not the basic principle in the size dependence of metabolic rate.

In mammals, the condition of thermoneutrality of environment, as applied in basal metabolism determination, amounts to minimize the energy expense for thermoregulation. The environmental temperature is so adjusted as to keep the body temperature normal without regulation, the heat arising as a by-product of the reactions in metabolism being sufficient to maintain body temperature. Only in conditions of non-thermoneutrality, muscular activity in the form of increased tension, shivering, etc. comes into play. This represents an excess superimposed over basal metabolic rate which is considerable indeed even in rather slight deviations from thermoneutrality, and which is measurable in appropriate experiments. This, however, does not concern basal respiration. Considering the fact that the decline of metabolic rate with increasing body size is a general phenomenon found also in animals without thermoregulation, it may be safely concluded that variations in the Q_{O_2} of musculature due to thermoregulation are not the factor responsible for the phenomenon in question.

b) It remains to be seen whether variations in the tissue respiration of skeletal muscle can account for the decline of weight-specific metabolic rate. Assuming a decrease of Q_{O_2} of musculature according to the surface or ¾-power rule, and considering the percentage of musculature in relation to total weight in small and large rats, according to Donaldson (1924, Table 124, p. 184), a simple estimate shows that the decline in muscular Q_{O_2} would not be sufficient to account for the decline in weight-specific basal metabolic rate of the entire animal.

The relation of Q_{O_2} of skeletal muscle to body size was recently studied by Bertalanffy and Estwick (1953). The Q_{O_2} of leg muscles of rats decreases, with increasing body size, with $\alpha = -.07$, *i.e.,* much less than would correspond to the surface or ¾-power rule. Interspecifically, the Q_{O_2} of skeletal muscle of adult mice (25–30 gm.) is similar to that of adult rats (over 300 gm.).

Thus there seems to be no indication that the decrease of weight-specific metabolic rate is to be explained by variations of the Q_{O_2} of musculature.

3) A third hypothesis often offered is that the mass of "metabolically active" organs decreases relatively with increasing body size. The high metabolism of small and young animals, as compared to the adult animals, would be based on the

fact that the inner organs which have a high oxygen consumption, are comparatively larger in the first (Kestner, 1934; Blank, 1934). Blank comes to the conclusion that the relatively larger size especially of the heart, kidney, intestinal tract and nervous system in smaller animals is responsible for their high oxygen consumption. However, already in 1942 (Bertalanffy, p. 198 ff.; 1951a, p. 249 ff.) we had shown by an analysis of the relative growth of the organs concerned that the latter is much too involved and too varying from one organ to the other, intraspecifically as well as interspecifically, that its combined effect is likely to result in a phenomenon so universal as the systematic decrease of weight-specific metabolic rate according to the simple power formula (on relative growth of inner organs of the rat; *cf.* Bertalanffy and Pirozynski, 1952).

A quantitative estimate may show that this factor cannot be the decisive one in the relative decrease in basal metabolism. As mentioned above, Field *et al.* (1939) have calculated summated tissue respiration for the mature rat. We have made a similar estimate for the 10-gm. rat, using Field's values for oxygen consumption per unit fresh weight for the individual organs, the wet weights of organs according to Donaldson (1924), and assuming a regression for Q_{O_2} of skeletal muscle, liver, lung, and heart as found in the present experiments. As calculated by Field *et al.*, summated tissue respiration gives 110 cc. O_2/hr. for the 150-gm. rat. The calculation carried through as mentioned above, gives a summated tissue respiration of *ca.* 8.7 cc. O_2/hr. for the 10-gm. rat. Since the basal metabolic rate of an animal of 150 gm. is *ca.* 165 cc. O_2/hr., that of an animal of 10 gm. *ca.* 25 cc. O_2/hr., summated tissue respiration as based upon Q_{O_2}-measurements in saline, accounts for 66% of basal metabolism in the mature rat, but only for 35% in the 10-gm. rat. Although such estimate is, of course, crude and over-simplified, it shows that *a*) the different proportion of inner organs in small and large animals does not account for the differences in their basal metabolic rates; and *b*) that respiration in a young animal must be considerably higher than the values obtained in saline.

4) Another possible correlation is that between tissue respiration and age. According to Hawkins (1928) who studied liver slices of three groups of rats, aged 3–21 days, one year, and 22 months, respectively, and Pearce (1936) who used liver, heart and kidney of two groups (4–9 and 50–60 weeks) of mice, Q_{O_2} values decrease with age. Although our experiments show a decrease of Q_{O_2} with increasing body size in some organs, they do not show a definite relation between Q_{O_2} and age. Our determinations are not in contradiction with those of the authors mentioned. However, one would expect that the organs which show the most conspicuous histological changes (deposits of lipofuscins) and are particularly involved in the process of aging, namely, brain and heart, should also show definite changes in respiration with increasing age. This is not the case.

5) Our experiments seem, therefore, to contradict explanations of the decline in basal metabolic rate with increasing size which are based upon factors lying in the tissues themselves, and active, therefore, also in tissue respiration as observed with the Warburg technique. It appears that the decline in basal metabolic rate depends upon regulative factors lying in the organism as a whole. Many such factors can be taken into account: hormonal and neural regulators, supply of oxygen and metabolites especially of the Krebs cycle, etc. Variations in the energy expense for minimal functional activity (heart, lungs, kidney, etc.) are naturally also to be taken into account.

Our conclusion that respiration as determined *in vitro* does not necessarily correspond to respiration *in situ*, is derived from a study of normal animals and tissues. It may be mentioned that similar conclusions result from the study of malignant tissues. Potter (1951), investigating the Krebs cycle in tumors, states (p. 569) that "experiments with whole animals using fluoroacetate to block citrate oxidation *in situ* suggest that the enzyme activity of tissues *in situ* may be different than either the homogenate or slice would indicate." It may be hoped, therefore, that further study of the factors modifying tissue respiration can throw light on the problem of regulation of metabolism under conditions of normal as well as pathological growth.

Taking, as a starting point, the metabolic level in a young animal which perhaps roughly corresponds to the metabolic rate as observed in media containing all necessary metabolites, such as serum or the solutions indicated by Krebs (1950), it would seem that tissue respiration is damped in a larger animal, to a level roughly corresponding to that obtained in saline. If this is the case, it is suggestive to remark that also the growth of organs is limited by organismic factors. The "growth potency" of tissues is not limited by factors lying in those tissues themselves. This is shown by the fact that tissues which would not grow within the organism, do so if removed from the organism and cultivated *in vitro;* similarly, if the steady state and the "balance of organs" are disturbed, as in regeneration and compensatory hypertrophy; and finally under pathological conditions, in malignant growth. It may be that the metabolic activity of organs is bridled, as it were, within the organism, so that the systemic decline of weight-specific metabolic rate with increasing size takes place; that the same is true for their growth potency; and that both factors are connected. Investigation of these factors may perhaps lead into a deeper insight on growth, normal as well as malignant.

Summary

1. The relation between tissue respiration and body size was investigated in the rat. Determinations of Q_{O_2} were made on heart, lung, liver, kidney cortex, brain cortex, diaphragm, and thymus of animals ranging from 9 gm. to 392 gm. body weight, including some determinations on fetuses and fetal tissues. A statistical evaluation of *ca.* 230 experiments is given.

2. The diaphragm is the only organ investigated to show a definite and significant correlation between rate of tissue respiration and body size. Liver and thymus show a break in the regression line which corresponds to a number of other characteristic changes in metabolism and growth.

3. The experiments do not show systemic differences in tissue respiration accounting for the decrease of total metabolic rate with increasing body size.

4. A comparison between intraspecific and interspecific size-dependence of tissue metabolism is made.

5. The current theories on the systematic decrease of weight-specific metabolic rate, as expressed in the surface or ¾ power rule, are discussed in the light of the experiments presented. It is shown that none of the explanations proposed (decline of total metabolic rate as based upon decrease of the rate of tissue respiration, upon thermoregulation, upon decrease of Q_{O_2} of musculature, upon the relative decrease of "metabolically active" organs, upon age) in consistent. It appears

that the decline in basal metabolic rate depends on regulative factors lying in the organism as a whole.

LITERATURE CITED

BENEDICT, F. G., 1938. Vital energetics. Carnegie Institution of Washington Publ., **503.**

BERTALANFFY, L. VON, 1938. A quantitative theory of organic growth. Inquiries on growth laws. II. *Human Biology,* **10**: 181–213.

BERTALANFFY, L. VON, 1942 and 1951a. Theoretische Biologie. Bd. II: Stoffwechsel, Wachstum. 1st ed. Berlin: Gebrueder Borntraeger. 2nd ed. Bern: A. Francke A. G.

BERTALANFFY, L. VON, 1951b. Metabolic types and growth types. *Amer. Nat.,* **85**: 111–117.

BERTALANFFY, L. VON, AND R. R. ESTWICK, 1953. Tissue respiration of musculature in relation to body size. *Amer. J. Physiol.,* **173**: 58–60.

BERTALANFFY, L. VON, AND J. KRYWIENCZYK, 1953. The surface rule in crustaceans. *Amer. Nat.,* **87**: 107–110.

BERTALANFFY, L. VON, AND I. MÜLLER, 1943. Untersuchungen ueber die Gesetzlichkeit des Wachstums. VIII. Die Abhaengigkeit des Stoffwechsels von der Koerpergroesse und der Zusammenhang von Stoffwechseltypen und Wachstumstypen. *Rivista di Biologia,* **35**: 48–95.

BERTALANFFY, L. VON, AND W. J. PIROZYNSKI, 1951. Tissue respiration and body size. *Science,* **113**: 599–600; **114**: 306–307.

BERTALANFFY, L. VON, AND W. J. PIROZYNSKI, 1952. Ontogenetic and evolutionary allometry. *Evolution,* **6**: 387–392.

BLANK, H., 1934. Tiergroesse und Stoffwechsel. *Pflügers Arch.,* **234**: 310–317.

BORGER, G., AND H. GROLL, 1926. Die individuellen Schwankungen der Sauerstoffatmung des ueberlebenden und entzuendeten Gewebes. *Krankheitsforschung,* **3**: 443–467.

BRODY, S., 1945. Bioenergetics and growth. New York: Reinhold Publishing Co.

CRANDALL, R. R., AND A. H. SMITH, 1952. Tissue metabolism in growing birds. *Proc. Soc. Exp. Biol. Med.,* **79**: 345–346.

DEUTSCH, W., AND H. S. RAPER, 1936. Respiration and functional activity. *J. Physiology,* **87**: 275–286.

DONALDSON, H., 1924. The rat. Second ed. Philadelphia: Memoirs Wistar Institute No. 6.

ELLIOT, K. A. C., 1948. Metabolism of brain tissue slices and suspensions from various mammals. *J. Neurophysiology,* **11**: 473–484.

FIELD, J., II, H. S. BELDING AND A. W. MARTIN, 1939. An analysis of the relation between basal metabolism and summated tissue respiration in the rat. I. The post-pubertal albino rat. *J. Cell. Comp. Phys.,* **14**: 143–157.

FÜSSER, H., AND F. KRÜGER, 1951. Vergleichende Versuche zur Atmungsphysiologie von *Planorbis corneus* und *Limnaea stagnalis* (Gastropoda Pulmonata). *Zeitschr. vergl. Physiol.,* **33**: 14–52.

GRAFE, E., 1925. Probleme der Gewebsatmung. *Dtsch. Med. Wschr.,* **51**: 640–642.

GRAFE, E., H. REINWEIN AND SINGER, 1925. Studien über Gewebsatmung. II. Die Atmung der überlebenden Warmblüterorgane. *Biochem. Zeitschr.,* **165**: 102–117.

GREGORY, P. W., AND H. GOSS, 1933. Glutathion concentration and hereditary body size. *J. Exp. Zool.,* **66**: 155–173.

HAWKINS, J. A., 1928. The metabolism of liver tissue from rats of different ages. *J. Gen. Phys.,* **11**: 645–647.

JANČAŘIK, A., 1948. Contribution to the knowledge of breathing of Cladocera *Daphnia pulex.* (Czech, English summary.) *Publ. Fac. Sci. Univ. Masaryk, ser. M 1, No. 305.*

KAYSER, CH., E. LEBRETON AND G. SCHAEFFER, 1925. Grandeur de la respiration des tissus et masse active au cours du développement des organismes. *C. R. Acad. Sci. (Paris),* **181**: 255–257.

KESTNER, O., 1934. Ueber die Oberflächenregel des Stoffwechsels. *Pflügers Arch.,* **234**: 290–301.

KITTEL, A., 1941. Sauerstoffverbrauch der Insekten in Abhängigkeit von der Koerpergrösse. *Zeitschr. vergl. Physiol.,* **28**: 533–562.

KLEIBER, M., 1941. Body size and metabolism of liver slices *in vitro.* *Proc. Soc. Exp. Biol. Med.,* **48**: 419–422.

KLEIBER, M., 1947. Body size and metabolic rate. *Physiol. Rev.*, **27**: 511–541.

KLEIBER, M., H. H. COLE AND A. H. SMITH, 1943. Metabolic rate of rat fetuses *in vitro*. *J. Cell. Comp. Phys.*, **22**: 167–176.

KRAMER, G., 1934. Der Ruheumsatz von Eidechsen und seine quantitative Beziehung zur Individuengrösse. *Zeitschr. vergl. Physiol.*, **20**: 600–616.

KREBS, A. H., 1950. Body size and tissue respiration. *Biochem. et Biophys. Acta*, **4**: 249–269.

KRÜGER, F., 1940. Die Beziehung des Sauerstoffverbrauches zur Körperoberfläche beim Schweinespulwurm (*Ascaris lumbricoides*). *Zeitschr. wiss. Zool.*, **152**: 547–570.

KRYWIENCZYK, J., 1952a. Körpergrösse, Körperzeiten und Energiebilanz. IV. Körpergrösse, O_2-Konsum und Kriechgeschwindigkeit bei Prosobranchiern. *Zeitschr. vergl. Physiol.*, **34**: 6–13.

KRYWIENCZYK, J., 1952b. Körpergrösse, Körperzeiten und Energiebilanz. V. Körpergrösse, O_2-Konsum und Kriechgeschwindigkeit bei Wasserpulmonaten. *Zeitschr. vergl. Physiol.*, **34**: 14–19.

LEBRETON, E., AND CH. KAYSER, 1926. La loi des tailles et la respiration des tissus *in vitro* chez les homéothermes. *C. R. Acad. Sci. (Paris)*, **183**: 397–399.

LIEBSCH, W., 1929. Ueber die Atmung einiger Heliciden. *Zool. Jb. Abt. Allg. Zool. u. Physiol.*, **46**: 161–208.

LUDWIG, W., AND J. KRYWIENCZYK, 1950. Körpergrösse, Körperzeiten und Energiebilanz. III. Der Sauerstoffverbrauch von Muscheln in Abhängigkeit von der Körpergrösse. *Zeitschr. vergl. Physiol.*, **32**: 464–467.

MARTIN, A. W., AND F. A. FUHRMANN, 1941. The relationship between basal metabolism and summated tissue respiration in the dog. *Amer. J. Phys.*, **133**: 379.

MÜLLER, I., 1943a. Untersuchungen zur Gesetzlichkeit des Wachstums. IX. Die Abhängigkeit der Atmung von der Körpergrösse bei *Dixippus morosus* und ihre Beziehung zum Wachstum. *Zeitschr. vergl. Physiol.*, **30**: 139–144.

MÜLLER, I., 1943b. Untersuchungen über die Gesetzlichkeit des Wachstums. X. Weiteres zur Frage der Abhängigkeit der Atmung von der Körpergrösse. *Biol. Zentralbl.*, **63**: 446–453.

NEEDHAM, J., 1934. Chemical heterogony and the groundplan of animal growth. *Biol. Rev.*, **9**: 79–109.

PATRUŠEV, V. J., 1937. On the inheritance of biochemical characters by animals and its relation to their growth. II. Glutathion concentration in the blood and differences in size in breeds of farm animals. *C. R. Acad. Sci. U. S. S. R., N. S.*, **14**: 573–577.

PEARCE, J. M., 1936. Age and tissue respiration. *Amer. J. Phys.*, **114**: 255–260.

PIROZYNSKI, W. J., AND L. VON BERTALANFFY, 1952. Is the rate of basal metabolism determined by tissue respiration? *Rev. Canad. Biol.*, **11**: 77–78.

POTTER, VAN R., 1951. Studies on the reactions of the Krebs citric acid cycle in tumor, with homogenates, slices, and *in vitro* technics. *Cancer Res.*, **11**: 565–570.

ROSENTHAL, O., AND D. L. DRABKIN, 1943. The cytochrome content of normal and neoplastic mammalian epithelium, and its correlation with body mass. *J. Biol. Chem.*, **150**: 131–141.

TERROINE, E. F., AND J. ROCHE, 1925. La respiration des tissus. I. Production calorique des homéothermes et intensité de la respiration *in vitro* des tissus homologues. *Arch. internat. physiol.*, **24**: 356–399.

UMBREIT, W. W., R. H. BURRIS AND J. F. STAUFFER, 1949. Manometric techniques and tissue metabolism. Burgess Publishing Co., Minneapolis.

WEINLAND, E., 1919. Beobachtungen über den Gaswechsel von *Anodonta cygnaea*. *Zeitschr. Biol.*, **69**: 1–86.

WEYMOUTH, F. W., J. FIELD, II AND M. KLEIBER, 1942. Relationship between body size and metabolism. *Proc. Soc. Exp. Biol. Med.*, **49**: 367–370.

WEYMOUTH, F. W., J. M. CRISMON, V. E. HALL, H. S. BELDING AND J. FIELD, II, 1944. Total and tissue respiration in relation to body weight. A comparison of the kelp crab with other crustaceans and with mammals. *Physiol. Zool.*, **17**: 50–71.

WILL, A., 1952. Körpergrösse, Körperzeiten und Energiebilanz. VI. Körpergrösse und O_2-Konsum bei Schaben und Asseln (Isopoden). *Zeitschr. vergl. Physiol.*, **34**: 20–25.

SUMMATION AND INHIBITION FOLLOWING CONTRALATERAL STIMULATION OF THE TARSAL CHEMORECEPTORS OF THE BLOWFLY

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In one of the efforts to arrive by behavioral methods at an understanding of the fundamental process of stimulation of tarsal chemoreceptors by unacceptable compounds it was discovered that the action of opposing stimuli, as for example sugar and alcohol, could be either ipsilateral or contralateral (Dethier, 1950). Normal proboscis extension in response to sugar could be prevented by adding propanol to sucrose solutions or by stimulating one leg with propanol alone while the opposite leg was exposed to sucrose. The fact that prevention of proboscis response could result from interaction in the central nervous system of opposing patterns of activity from opposite legs directed attention to the existence of central inhibtion in this insect.

In addition to providing information relative to the interaction of *unlike* stimuli, the early experiments yielded data which pointed to the existence of central summation following stimulation by *like* stimuli. However, another series of experiments in which contralateral stimulation by like stimuli was investigated for the olfactory sense (Dethier, 1952a) suggested that the observed results might be simply an expression of a statistical bias introduced by doubling the available number of receptors. When rejection thresholds for pentanol vapor were compared in flies with a single antenna and those with both antennae, it was observed that thresholds were lower for bilateral stimulation than for unilateral; but the decrease of bilateral over unilateral was never greater than could be satisfactorily accounted for on a simple probability basis (*cf.* Smith and Licklider, 1949). Thus, while these tests did not negate the existence of true neural contralateral summation, they did not necessarily affirm it. Since the experiments were designated to measure the per cent response at only one or two test concentrations rather than to ascertain values for complete threshold frequency distribution, a rigorous statistical analysis was not possible. Nor did it appear feasible, because of the difficulty of controlling vapor concentrations, to attempt to resolve the problem with the antennal preparation. Tarsal chemoreceptors are clearly more amenable to complete analysis. Consequently, the investigation described herein was undertaken to extend earlier tarsal as well as olfactory data in an effort to permit a clearer statement of the status of contralateral summation and inhibition in the response of the blowfly to chemicals.

Methods

The solution of two immediate problems was undertaken: (1) to determine whether or not simultaneous stimulation of opposite legs by like stimuli sums in the central nervous system; (2) to determine to what extent unlike stimuli inhibit each

other. Experimental methods fell into two categories. In the first, tests were conducted with one-legged and two-legged flies. These insects were prepared as follows. One- to two-day old flies (*Phormia regina*) from a stock culture were anesthetized with carbon dioxide and attached by the wings to wax-tipped applicator sticks. In the case of one half of the flies, all legs except one prothoracic were fastened into the wax. Of this group of flies, one half had the right prothoracic leg free and the other half, the left. In the remaining group of flies all legs except the prothoracic pair were immobilized. Measurements were then made of the responses of one-legged and two-legged flies to sucrose, propanol in water, propanol in 0.1 *M* sucrose, and HCl in water.

In the second series of experiments the responses of two-legged flies were tested in partitioned dishes. The vessels used were Stender dishes 45 mm. in diameter with a cover glass 40 mm. long fastened on edge across the middle with paraffin. The top of the cover glass was set flush with the top edge of the dish. A different solution could now be poured into each half of the dish, care being taken to insure that the liquids rose only to such a level as to make concave rather than convex menisci at the partition.

With this device it was possible to stimulate opposite prothoracic legs of a fly simultaneously with different solutions by causing him to straddle the partition. In practice, execution of a test required the utmost delicacy and the patience of Job. A captive fly suspended in air had a tendency to keep its legs crossed or flexed at the tibio-tarsal joint. A proper test cannot be made until both legs are extended and spread apart. When a fly had assumed this position, it was lowered gently over the partition. Care had to be exercised that both legs touched the respective solutions at approximately the same moment and that the partition was not grasped by either leg. Under favorable conditions, proboscis extension, if adequate stimulation was supplied, occurred in the usual manner.

This technique was employed to test unilateral versus bilateral stimulation by a single compound and to investigate the effects of opposing stimuli acting simultaneously on opposite legs. In the first instance, unilateral stimulation, by sucrose, for example, was accomplished by forcing the fly to straddle a partition which separated a sucrose solution from pure water. For bilateral stimulation sucrose was placed on both sides of the partition. Similarly, the stimulating effects of various combinations of sucrose, water, HCl, NaCl and propanol were investigated. The concentrations of HCl and propanol chosen for stimulation were those which, under normal conditions of testing, would yield responses well above the 50% level. The concentration chosen for NaCl was nearer to the 50% level. The number of flies used in each experiment varied from 60 to 800.

In every case, except where noted otherwise, the flies were random sampled, *i.e.*, a different group was tested at each concentration in the series, and the per cent which responded was noted. Median threshold values were calculatd according to the method of Bliss (1938) (*cf.* also Dethier and Chadwick, 1948).

Results

Unilateral versus bilateral stimulation by sucrose

Originally a straightforward comparison of the sucrose thresholds of one-legged and two-legged flies was made. In these experiments the flies were tested

in ascending order; that is, each fly was exposed successively to each concentration of the test solution, beginning with a subliminal concentration, until one was reached which elicited a proboscis extension. Since there was a real possibility that one-legged and two-legged flies were not strictly comparable, *i.e.*, that the number of legs immobilized might affect the general behavior of the fly, additional tests were run with two-legged flies, one of whose legs was tethered with a fine silken thread. Each such fly was tested with the tethered leg suspended above the solution (unilateral stimulation) and then with both legs in contact with the solution (bilateral stimulation). Finally the partitioned dish, as already described, was employed. This time the experiments were run in random rather than ascending series. As an added precaution and to insure accuracy of results most of the experiments with partitioned dishes were coded so that the experimenter was unaware of the contents of the dishes.

Regardless of the method of testing employed the results were similar (Table I). The unilateral threshold was always significantly higher than the bilateral threshold (Fig. 1). In these experiments, the absolute values obtained from random testing are not directly comparable with the values obtained from ascending testing because it was not possible to use flies of uniform age and nutritional background throughout the entire study. When these factors are controlled, ascending thresholds are higher than random thresholds (*cf.* Dethier, 1952b).

Contralateral stimulation by unlike compounds

In order first to survey the situation with regard to such representative unacceptable compounds as NaCl, HCl, and propanol, spot tests were undertaken

TABLE I

Comparison of bilateral and unilateral thresholds of response of Phormia regina to sucrose, HCl, and propanol†

Compound and technique of testing‡	Conc. at which 50% of flies respond	Log median ±2.575 S. E.	a + S. E.	b + S. E.	$\bar{x}$	No. of insects tested
Sucrose, one leg, ascending	0.0037	−2.433 ±0.191	5.140 ±0.050	0.713 ±0.082	−2.237	173
Sucrose, two legs, ascending	0.0018	−2.749 ±0.149	5.387 ±0.055	1.163 ±0.122	−2.416	173
Sucrose/water (=1 leg)	0.1010	−0.996 ±0.515	4.326 ±0.078	0.640 ±0.105	−1.993	360
Sucrose/sucrose (=2 legs)	0.0200	−1.699 ±0.258	4.672 ±0.021	0.812 ±0.030	−2.103	360
*Sucrose/water (=1 leg)	0.0177	−1.930 ±0.258	4.900 ±0.045	0.474 ±0.067	−2.141	800
*Sucrose/sucrose (=2 legs)	0.0021	−2.670 ±0.149	5.308 ±0.049	0.991 ±0.096	−2.359	800
Propanol in water, 1 leg	0.607	−0.217 ±0.156	5.037 ±0.073	1.206 ±0.137	−0.186	360
Propanol in water, 2 legs	1.05	0.020 ±0.162	4.734 ±0.068	1.160 ±0.121	−0.209	420
Propanol/water (=1 leg)	1.34	0.128 −0.170	5.011 −0.182	2.770 −0.589	0.132	60
Propanol in 0.1 *M* sucrose						
1 leg	0.59	−0.232 ±0.098	5.115 ±0.107	2.867 ±0.369	−0.192	200
2 legs	0.95	−0.021 ±0.098	4.832 ±0.105	2.770 ±0.351	−0.082	200
Propanol/0.1 *M* sucrose, random	1.23	0.088 ±0.165	5.077 ±0.083	1.332 ±0.253	0.146	240
Propanol/0.1 *M* sucrose, ascending	1.076	0.032 ±0.155	5.132 ±0.134	2.254 ±0.311	0.090	120
HCl in 0.1 *M* sucrose, 1 leg	1.041	−1.391 ±0.163	4.877 ±0.068	1.107 ±0.147	−1.502	70
HCl in 0.1 *M* sucrose, 2 legs	0.051	−1.298 ±0.109	4.773 ±0.069	1.441 ±0.142	−1.456	70

* As a check against the method all solutions in these series were coded.

† The 4th, 5th, and 6th columns give the calculated values for a, b, and $\bar{x}$ in the equation $Y = a + b(X - \bar{x})$, which is the regression of per cent flies responding, Y, expressed as probits on log concentration, X.

‡ Unless stated otherwise all tests represent random sampling. The slanting line symbolizes a divided dish. For explanation of different sucrose thresholds see text.

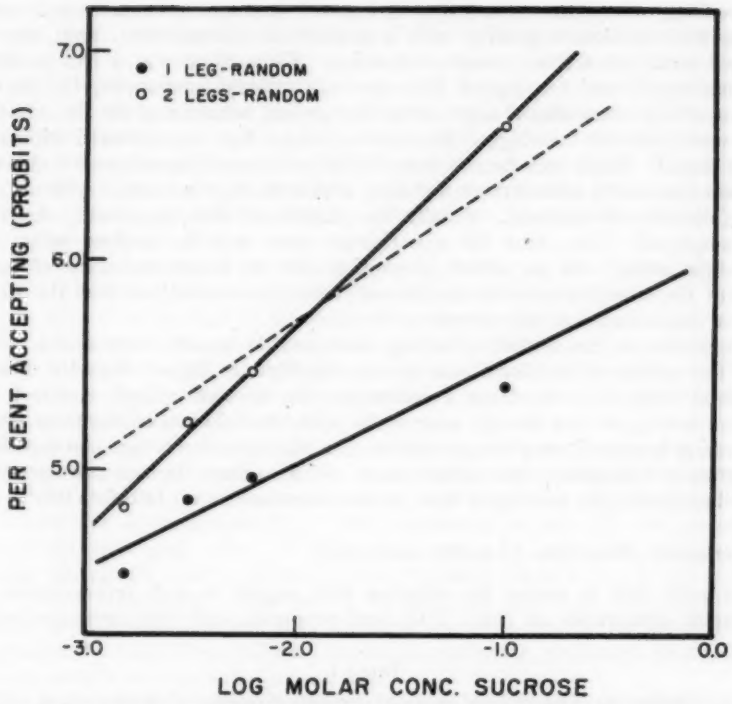

FIGURE 1. Comparison of the distribution of acceptance thresholds for sucrose, as a function of concentration, for flies stimulated unilaterally or bilaterally. The broken line represents the theoretical distribution of bilateral thresholds (two-legged flies) calculated from the expression $1-q^2$ where q equals the fraction of the population of one-legged flies not responding.

by means of a series of partitioned dishes. As had been done earlier in the olfactory studies (Dethier, 1952a) the procedure was adopted of selecting a single concentration at which measurements were made of the change in per cent of the population responding as a different number of appendages was stimulated. This course of action was followed for the sake of simplicity in lieu of varying the concentration to ascertain what strength of stimulus was required to elicit 50% response (median threshold) under each changed experimental condition. However, the quantitative information yielded by such spot testing cannot be utilized to its fullest extent in the absence of additional experiments because there is no way of knowing without further testing what relation the slopes of the threshold distribution curves of one-legged and two-legged flies bear to each other. It will be shown further on that the results of spot tests are substantiated by data from more detailed experiments.

The results obtained with the various spot combinations are arranged in series of pairs (Table II) for purposes of comparison. The following numbers cor-

respond to the comparison numbers in the table, and the nature of the comparison is further indicated here by the symbols in parentheses. A slanting line indicates the partition dividing the two solutions, *s* signifies sucrose, and *P* signifies a repellent compound.

I (H_2O/s vs. P/s). All three unacceptable compounds can prevent a response to sucrose even when acting on the leg opposite to that leg which is being stimulated by sucrose. This is shown by the increase in per cent rejection over that recorded when water alone is paired with sucrose.

TABLE II

Comparison of mean rejection under different conditions of contralateral stimulation

Comparison no.	2 *M* propanol vs. 0.1 *M* sucrose and H_2O		1 *M* NaCl vs. 0.1 *M* sucrose and H_2O		0.1 *M* HCl vs. 0.1 *M* sucrose and H_2O	
	Condition of test	Mean % rejection	Condition of test	Mean % rejection	Condition of test	Mean % rejection
I	H_2O/s	47.2	H_2O/s	58.7*	H_2O/s	46.6
	P/s	70.9	NaCl/*s*	60	HCl/*s*	65.3
II	$H_2O/P+s$	87.2	H_2O/NaCl+*s*	65.6*	H_2O/HCl+*s*	91.3
	s/*P*+*s*	54.5	*s*/NaCl+*s*	53	*s*/HCl+*s*	73.3
III	*s*/*P*+*s*	54.5	*s*/NaCl+*s*	53*	*s*/HCl+*s*	73.3
	P/*P*+*s*	94.5	NaCl/NaCl+*s*	66	HCl/HCl+*s*	99
IV	$H_2O/P+s$	87.2	H_2O/NaCl+*s*	65.6*	H_2O/HCl+*s*	91.3
	P/*s*	70.9	NaCl/*s*	60	HCl/*s*	65.3
V	*P*/*s*	70.9	NaCl/*s*	60*	HCl/*s*	65.3
	P/*s*+*P*	94.5	NaCl/*s*+NaCl	66	HCl/*s*+HCl	99
VI	*P*+*s*/*P*	94.5‡	NaCl+*s*/NaCl	66*	HCl+*s*/HCl	99†
	P+*s*/H_2O	87.2	NaCl+*s*/H_2O	65.6	HCl+*s*/H_2O	91.3
n	110		160		150	

* There is no significant difference between the means compared in these pairs.
† Difference is significant at $P = 0.02$.
‡ Difference is significant at $P = 0.05$. All other differences are highly significant.
n = number of flies tested in each paired comparison.

II ($H_2O/P + s$ vs. $S/P + s$). An appreciably lower rejection is obtained with sucrose on both sides of the partition and a repellent compound on one side only. This result is in agreement with the fact that bilateral stimulation by sucrose is followed by greater acceptance than is unilateral stimulation.

III ($s/P + s$ vs. $P/P + s$). This comparison seems to show that there is a greater rejection when two legs are stimulated by unacceptable compounds than when a single leg is stimulated. This apparent summation is attributable, at least in part, to the difference with respect to sucrose stimulation. Note that in one member of the pair there is sucrose on both sides of the partition while in the other it is on one side only. This result agrees with II above.

IV ($H_2O/P + s$ vs. P/s). Greater rejection is obtained when opposing compounds stimulate the same leg than when they act independently on opposite legs. This effect may be due either to some inhibiting action of the repellent compound on the sugar receptors over and above its independent effect (see I) or to some "loss" of repellent effectiveness as a result of looser central integration when opposing stimuli act on different sides.

V (P/s vs. $P/s + P$). There is greater rejection when the unacceptable compound acts on both legs instead of on one. Part of the observed difference may result from the fact that in one dish P and s are acting independently as well as contralaterally and that ipsilateral repellency is greater than contralateral repellency as shown in IV above.

VI ($P + s/P$ vs. $P + s/H_2O$). When the sugar is identical in both tests (here sucrose is on one side only), the repellency is slightly but consistently higher with the repellent on both sides of the partition. This experiment shows that for unacceptable compounds the bilateral threshold is lower than the unilateral threshold.

Unilateral versus bilateral stimulation by unacceptable compounds

As has already been pointed out, a critical comparison of unilateral versus bilateral thresholds must be based upon complete threshold frequency distribution curves and not merely upon responses to a single selected concentration. Consequently, as a check on the foregoing results, especially comparison VI, and with propanol and HCl as models, tests were run over complete concentration ranges. With propanol, tests were run with one-legged flies, two-legged flies, and with partitioned dishes. Propanol was tested with and without the addition of sucrose. With HCl, tests were run with one-legged and two-legged flies. In each case 0.1 M sucrose was mixed with the HCl. Data are summarized in Table I. Three facts emerge clearly from an examination of these data: (1) there is no statistically significant difference between unilateral and bilateral thresholds for either propanol or HCl; (2) nevertheless, unilateral thresholds have a slight but consistent tendency to be lower than bilateral thresholds; (3) there is no significant difference of threshold for propanol in water and in sucrose.

Discussion

As early as 1938 the experiments (Table 16) of Imamura in which tarsal receptors of the fly *Sturmia sericariae* Cornalia were stimulated by sugar had given an intimation of an increased sensitivity to bilateral stimulation over unilateral. Studies of olfactory thresholds of *Phormia regina* (Dethier, 1952a) had yielded data which suggested the possibility of a precise quantitative relationship between bilateral and unilateral thresholds. It is now clear that such quantitative relationships exist. But although it can be proven that for any individual the bilateral thresholds for certain compounds are significantly lower than the unilateral thresholds, it is by no means certain that the increased sensitivity is attributable to contralateral summation in the usually accepted sense.

In the analogous case, where comparisons have been made between monocular and binocular vision, Pirenne (1943) and Bárány (1946a, 1946b) have pointed

out that the experimental procedure by its very nature assures that the two eyes will see more clearly than one. "Let us assume that the visual acuities (or other thresholds) of both eyes fluctuate independently of one another . . . and that the instantaneous thresholds for monocular vision have the same distribution in both eyes, . . . then if the one eye alone has the chance a of seeing the symbol, both eyes together have the chance $2a - a^2$. As a is smaller than 1, this expression will always be greater than a—that is to say, two eyes will be able to see better than one solely as a result of random combination" (Bárány, 1946b, p. 127). And, as Smith and Licklider (1949) go on to state, the same source of bias is inherent in the procedure as applied to the determination of thresholds in other sense modalities. The idea can be clarified still further by quoting from the study of hearing by these authors (p. 279). "In order to estimate the magnitude of the bias, it is necessary to define the null condition under which we should say that there is no binaural summation. We can imagine, for this purpose, two monaural listeners, one with only a right ear, the other with only a left ear. The two listeners have no means whatsoever of communicating with each other, but both report to the same experimenter. To obtain measures of monaural and 'binaural' sensitivity, the experimenter tests the two listeners separately (successively), then together, in the latter instance recording a positive response whenever *either* listener reports hearing the stimulus tone. It need hardly be said that we do not propose this schema as a psychophysiological hypothesis. There is of course no doubt that the two ears of a single listener do send their messages to a common center in which true summation may occur. Evidence of interaural interaction has been found in psychophysical experiments on sound localization and on masking and in electrophysiological observations at the levels of the cochlear nucleus and the temporal lobe. The schema is therefore a 'null hypothesis' [of supplementation] which a given set of data should lead us to reject before we say that the data constitute evidence of true binaural summation."

The applicability of this hypothesis of supplementation to the present case is easily tested. Let us assume that x number of sucrose receptors on the tarsi must be activated in order to insure a response, that is, that there is intra-leg summation. Then at the median acceptance threshold value 50% of the flies have x or more receptors acting. The probability at this concentration that a fly (x or more active receptors) will be in the half of the population which is responding is 50%. This probability can be raised either by increasing the concentration or by increasing the number of available receptors (n). If n is doubled, the probability of a response occurring increases. The increase can be calculated from the expression $1 - q^2$ where q is equal to the fraction not acting at the median concentration. Accordingly, the concentration which elicits a response from 50% of the one-legged flies should elicit a response from 75% of the two-legged flies if there is no interaction between the two legs. In the present experiments this expectation is realized. As a visual examination of Figure 1 shows, the response of the two-legged flies is not greater, except at the highest concentrations, than that which would be predicted on a probability basis from the behavior of one-legged individuals. The difference between the expected line (calculated from the line for one-legged thresholds from the expression $1 - q^2$ where q = the fraction not responding) and the line describing actual two-legged thresholds is not significant. Thus the data do not constitute evidence of true contralateral summation. Never-

theless, as was pointed out in the case of olfactory responses (Dethier, 1952a), the results represent from the point of view of the integrated organism a behavioral summation of no little importance.

The action of unacceptable compounds (propanol, HCl, NaCl) on one-legged and two-legged flies appears at first to follow an entirely different pattern from that observed with sucrose. Bilateral thresholds are not lower than unilateral

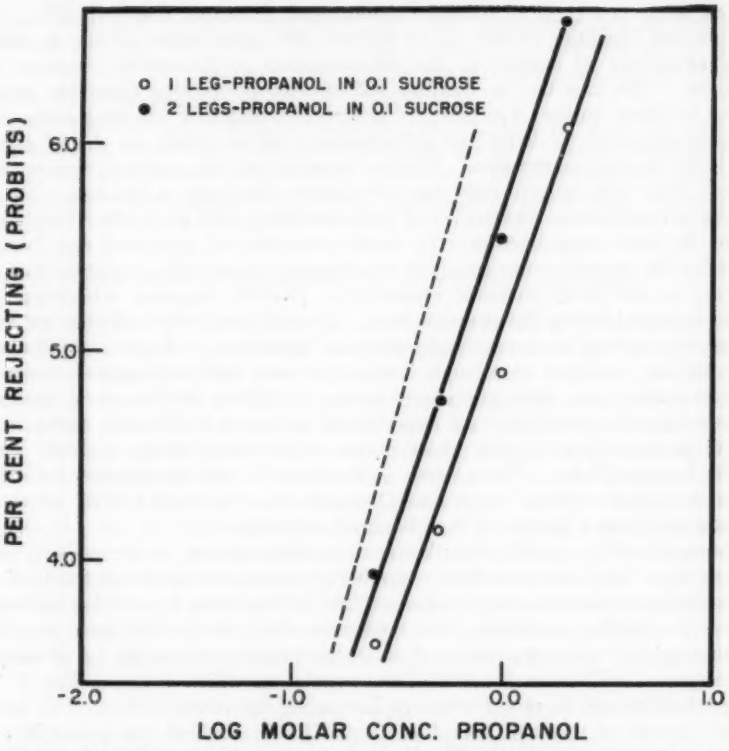

FIGURE 2. Comparison of the distribution of rejection thresholds for propanol, as a function of concentration, for flies stimulated unilaterally or bilaterally. The broken line represents the theoretical distribution of bilateral thresholds (two-legged flies) calculated from the expression $1\text{-}q^2$ where q equals the fraction of the population of one-legged flies not responding.

thresholds as might have been expected and as the spot tests (comparison VI) show. Actually, data from the spot tests and from complete concentration tests are in agreement, and supplementation occurs in much the same manner as it does with sucrose. The following explanation of the one-legged and two-legged fly experiments should clarify this point: (1) less sucrose is required to elicit a response from two-legged than from one-legged flies; (2) in most of the experiments with propanol and HCl, the test solutions contained 0.1 *M* sucrose regardless

of the number of legs to be stimulated; therefore, since the sucrose "sums" contralaterally, there was actually a greater effect for the propanol and HCl to overcome in two-legged flies than in one-legged flies; (3) if propanol and HCl "sum" as sucrose does, the greater sucrose effect would be overcome with the same concentrations of propanol or HCl in the one-legged and the two-legged flies; in other words, the rejection thresholds should be the same regardless of the number of legs stimulated; (4) if neither propanol nor HCl "sums," then a greater concentration of these compounds would be required on a two-legged fly to overcome the increased sucrose effect, and, therefore, propanol and HCl thresholds should be higher with two-legged than with one-legged flies.

A comparison of thresholds of one-legged and two-legged flies for unacceptable compounds reveals no statistically significant difference between the two although a slight but consistent tendency for the bilateral threshold to be higher has been commented upon. It would be expected, however, that if this tendency were truly an expression of a failure to sum or supplement, as outlined under (4) in the preceding paragraph, the difference would be of the same order of magnitude as that found with unilateral and bilateral sucrose thresholds but of the opposite sign. Obviously the observed difference is too small. The alternative suggestion, that unacceptable compounds do indeed "sum" in a manner similar to sucrose, though possibly not to the same extent, fits the data with fewer contradictions. This interpretation is strongly supported by the spot tests in comparison VI ($P + s/P$ vs. $P + s/H^2O$) where the sugar concentrations are controlled in such a way that the difference in unilateral and bilateral thresholds shows up directly.

Furthermore, this interpretation agrees with the results obtained in olfactory tests where the unacceptable compound pentanol was found to stimulate a fly with two antennae more effectively than it did a fly with one antenna (Dethier, 1952a). The reason that a threshold difference could be demonstrated directly in the olfactory tests is explained by the fact that the pentanol vapor was not counteracting the effect of an acceptable compound. The pentanol rejection threshold represented the concentration necessary to drive the flies away from light. When one antenna was amputated in these experiments, the number of receptors being stimulated by light was in no way affected. On the other hand, immobilization of one leg in the tarsal experiments not only reduced the number of receptors being stimulated by the unacceptable compound but also the number affected by the acceptable compound.

The foregoing considerations involving tarsi have been based principally upon those cases in which the unacceptable compounds were mixed with sucrose. An examination of Table I will show that comparable results were obtained with propanol when sucrose was absent from the test solutions. Determinations of the rejection thresholds of thirsty flies for propanol in water gave unilateral and bilateral threshold values which were not significantly different from each other. By applying the same reasoning to data obtained from water mixtures as was applied above to data relating to sucrose mixtures, one arrives at the conclusion that just as there is supplementation of response to sucrose, so also is there supplementation with water. Since the concentration of water cannot be altered, direct demonstration of the phenomenon of supplementation is impossible in this case.

In the sense that propanol, HCl, and NaCl alter the normal pattern of activity from tarsal receptors, they may be said to be stimulating. While a conclusion of this sort is hardly unexpected insofar as the electrolytes are concerned, some find it difficult to envision alcohol as a stimulus to sensory structures. Yet observations of the reactions of individual flies show that in solutions containing alcohol there is agitated movement of the feet, even attempts at withdrawal, and that all gradations exist between absolute failure to extend the proboscis, complete extension followed immediately by exceptionally rapid withdrawal, and partial rather hesitant extension. In other studies, many species of insects have been shown to be stimulated by alcohols. The ovipositors (which are insensitive to water) of certain hymenopterous parasites and of the cricket (*Gryllus*) are excited by inorganic electrolytes and aliphatic alcohols. Application of threshold concentrations results in characteristic movements of the organ (Dethier, 1947, 1951). The beetle *Laccophilus* is stimulated to greater swimming activity by alcohols (Hodgson, 1951). *Phormia* is stimulated via the antennae to greater flying activity by alcohol vapors (Dethier and Yost, 1952), and *Drosophila* (Reed, 1938) is attracted by low concentrations of short-chain aliphatic alcohols. On the other hand, it is also known that short exposure to high concentrations or prolonged exposure to lower concentrations of alcohols will result in narcosis. What may be narcosis of tarsal sugar receptors has been observed in *Phormia* (cf. Dethier, 1951).

From the neurophysiological point of view the action of a compound like alcohol could be either excitatory or depressant, or excitatory at one concentration and depressant at another. Peripheral inhibition of activity in sense organs by a stimulus as a means of "stimulating" an animal has been recorded sporadically. Granit (1947) and others have described fibers of the retina in which there is commonly spontaneous activity which may be depressed by illumination. Löwenstein and Sand (1940) have reported that spontaneous discharges from single fibers from the horizontal ampulla of the isolated labyrinth of *Raja* are increased by ipsilateral stimulation and inhibited by contralateral stimulation.

Ultimately the revelation of the nature of those events which are occurring peripherally in *Phormia* at the time of stimulation can be realized probably only through the agency of electrophysiological techniques. However, the experiments reported here foreshadow to some extent the nature of these events. Data from experiments with divided dishes do not in themselves prove that unacceptable compounds and sucrose act upon different receptors, but taken in conjunction with other experiments they lend strong support to the idea that there are at least two types of tarsal chemoreceptors. Admittedly, it could be argued that only a single type of receptors need be postulated to explain the facts. An argument along these lines, which could apply equally well to ipsilateral and contralateral stimulation by opposing stimuli, would require that sucrose stimulate the receptors and that alcohol depress them. It would require further that there be spontaneous activity from the receptors and that any decrease in the basal level of discharge from one leg could offset in the central nervous system the increased activity from the opposite leg which was undergoing stimulation by sucrose. According to this scheme, however, any factor which could depress spontaneous activity in one leg should be able to prevent proboscis response when the opposite leg is stimulated by sucrose. Amputating a leg would be a most effective means of obliterating spontaneous activity. Yet, when

this is done, stimulation of the remaining prothoracic leg by a threshold amount (for unilateral stimulation) of sucrose elicits the usual proboscis response.

Another experimental result which is not in accord with the one-receptor-type hypothesis is the quantitative difference between supplementation with sucrose and supplementation with unacceptable compounds. With sucrose the difference between unilateral and bilateral thresholds is considerable; with unacceptable compounds it is difficult to demonstrate, even in the absence of such disturbing influences as water and sucrose (*cf.* spot test comparison VI). It would be expected that supplementation would be of equal value with acceptable and unacceptable compounds if these were acting on the same population of receptors.

In view of the objections just stated, it is difficult to reconcile the available data with a one-type-receptor hypothesis. All of the results reported are more consistent with the idea that sucrose and unacceptable compounds act on different populations of receptors. It is to be hoped that the tarsal receptors may eventually yield to attack by electrophysiological methods.

Summary

Acceptance thresholds of the tarsal chemoreceptors of the blowfly, *Phormia regina*, for sucrose and rejection thresholds for HCl, NaCl, and propanol have been determined. Comparisons were made of the thresholds of one-legged and two-legged flies. Comparisons were also made of rejection thresholds determined (a) on one leg exposed to a mixture of sucrose and unacceptable compound and (b) on two contralateral legs, one of which was exposed to sucrose alone and the other to an unacceptable compound alone, the exposure being simultaneous. The following results were obtained:

(1) The bilateral threshold for sucrose is lower than the unilateral threshold.
(2) For sucrose the decrease of bilateral over unilateral threshold is never greater than can be satisfactorily accounted for on a simple probability basis.
(3) Unacceptable compounds terminate the response to sucrose even when applied to the leg not receiving sucrose, *i.e.*, they can act contralaterally as well as ipsilaterally.
(4) There is a greater rejection when two opposing stimuli act on one leg than when they stimulate two contralateral legs.
(5) Unacceptable compounds also "sum" contralaterally.
(6) Supplementation also occurs in the response of thirsty flies to water.
(7) The prevention of proboscis extension to water and sucrose by unacceptable compounds is predominantly a central phenomenon.
(8) Available evidence favors the interpretation that there are at least two distinct sets of receptors on the tarsi, sugar receptors and non-sugar receptors.

Literature Cited

Bárány, E., 1946a. A theory of binocular visual acuity and an analysis of the variability of visual acuity. *Acta Ophthal. Kbh.*, **24**: 63–92.

Bárány, E., 1946b. Some statistical observations on the methods in threshold determinations in general with particular regard to determination of visual acuity and subliminal addition. *Acta Opthal. Kbh.*, **24**: 113–127.

BLISS, C. I., 1938. The determination of the dosage-mortality curve from small numbers. *Quart. J. Pharm. and Pharmacol.*, **11**: 192–216.

DETHIER, V. G., 1947. The response of hymenopterous parasites to chemical stimulation of the ovipositor. *J. Exp. Zool.*, **105**: 199–208.

DETHIER, V. G., 1950. Central summation following contralateral stimulation of tarsal chemoreceptors. *Fed. Proc.*, **9**: 31–32.

DETHIER, V. G., 1951. The limiting mechanism in tarsal chemoreception. *J. Gen. Physiol.*, **35**: 55–65.

DETHIER, V. G., 1952a. The relation between olfactory response and receptor population in the blowfly. *Biol. Bull.*, **102**: 111–117.

DETHIER, V. G., 1952b. Adaptation to chemical stimulation of the tarsal receptors of the blowfly. *Biol. Bull.*, **103**: 178–189.

DETHIER, V. G., AND L. E. CHADWICK, 1948. The stimulating effect of glycols and their polymers on the tarsal receptors of blowflies. *J. Gen. Physiol.*, **32**: 139–151.

DETHIER, V. G., AND M. T. YOST, 1952. Olfactory stimulation of blowflies by homologous alcohols. *J. Gen. Physiol.*, **35**: 823–839.

GRANIT, R., 1947. Sensory mechanisms of the retina. Oxford Univ. Press, London.

HODGSON, E. S., 1951. Reaction thresholds of an aquatic beetle *Laccophilus maculosus* Germ., to salts and alcohols. *Physiol. Zool.*, **24**: 131–140.

IMAMURA, S., 1938. Studies on the chemical susceptibility of the Kyôsofly, *Sturmia sericariae* Cornalia. *Bull. Imp. Sericultural Exp. Sta. (Tokyo)*, **9**: 219–269.

LÖWENSTEIN, O., AND A. SAND, 1940. The mechanism of the semicircular canal. A study of the responses of single-fiber preparations to angular accelerations and to rotation at constant speed. *Proc. Roy. Soc. London, Ser. B*, **129**: 256–275.

PIRENNE, M. H., 1943. Binocular and monocular threshold of vision. *Nature*, **152**: 698–699.

REED, M. R., 1938. The olfactory responses of *Drosophila melanogaster* Meigen to the products of fermenting banana. *Physiol. Zool.*, **11**: 317–325.

SMITH, M. H., AND J. C. R. LICKLIDER, 1949. Statistical bias in comparisons of monaural and binaural thresholds: binaural summation or binaural supplementation. *Psychol. Bull.*, **46**: 278–284.

MATING TYPES IN TETRAHYMENA[1]

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The potentialities of *Tetrahymena* as a genetic tool have long been considered by those working with this ciliated protozoan. Its value stems from the fact that it is one of the few animal cells that can be grown in a defined medium. The failure of laboratory strains to reproduce sexually has been the chief obstacle to its use in genetic studies. The recent discovery of conjugation (selfing) in several strains (AA) taken from the Ann Arbor area (Elliott and Nanney, 1952) stimulated a further search for wild strains in which mating types might be found. These have been found and the purpose of this paper is to describe certain aspects of their physiology and cytology, and to discuss how they may be handled in the laboratory with a view to their use in genetic studies.

Materials and Methods

One hundred twenty-seven clones of *Tetrahymena* sp. (probably *T. pyriformis*) were established from 15 different fresh water habitats in the Woods Hole, Massachusetts area (Elliott and Gruchy, 1952). Three of these turned out to be selfers whereas all others did not conjugate within the clone. Cross-matching in all combinations finally yielded a pair of mating clones which came from one pond. Sixteen of the 17 other clones from this source mated readily with either one or the other of these two. Clone 52 from another pond conjugated with both of the first two. These three have been designated WH (Woods Hole) strains 6, 14, and 52, mating types I, II, and III, respectively. Throughout this paper strains 6, 14, and 52 will be referred to only as mating types I, II, and III. Two strains which will mate with types I, II, and III have also been isolated from a lake in northern Minnesota (Park Rapids area). One of these is another mating type I which conjugates with types II and III; the other, a type II, mates with types I and III.

All clones were established in axenic cultures by placing several cells from a bacterized culture (0.1% Cerophyll seeded with *Aerobacter aerogenes*) in a depression slide containing a mixture of penicillin G and streptomycin (250 γ/ml. of each) in the stock medium. The stock medium contains Bacto-tryptone 5 gm., Bacto-proteose-peptone 5 gm., sodium acetate 1 gm., thiamine HCl 0.002 gm., yeast extract 0.1 gm., KH_2PO_4 1 gm., and 1000 ml. of glass-distilled water. Its pH is adjusted to 7.2 with NaOH. After approximately 12 hours, single-cell isolations were made into several depressions containing the antibiotic medium. Twenty-

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four hours later, during which time growth of the protozoan had occurred (about 10 divisions), the entire drop was transferred to a test tube containing the stock medium without the antibiotics. Only rarely did this procedure fail to eliminate contaminating organisms as verified by the customary sterility tests.

Stock lines have been maintained continuously in this medium and, except where otherwise stated, all experiments are conducted with organisms grown in this medium. Loop inoculations reach peak growth in about 7 days and the cells remain viable for two or more months, thus permitting the handling of large numbers of clones without frequent sub-culturing.

Experimental

I. Nutrition

In determining the nutritional requirements of the mating types, the eleven amino acids and seven B-vitamins essential for the long-established strain E (Elliott, 1949, 1950) were tested by single omissions from the complete defined medium that supports normal growth of strain E (Table I). The results of this

Table I

Defined medium

	Micrograms per milliliter
L-Arginine·HCl	150
L-Histidine·HCl·H_2O	110
DL-Isoleucine	100
DL-Leucine	140
L-Lysine·HCl·H_2O	70
DL-Methionine	70
DL-Phenylalanine	100
DL-Serine	180
DL-Threonine	180
L-Tryptophan	120
DL-Valine	60
Asparagine	85
Dextrose	1000
Sodium Acetate	1000
$MgSO_4 \cdot 7H_2O$	10.0
K_2HPO_4	100.0
$Zn(NO_3)_2 \cdot 6H_2O$	5.0
$FeSO_4 \cdot 7H_2O$	0.5
$CuCl_2 \cdot 2H_2O$	0.5
Uracil	25.0
Cytidylic acid	25.0
Guanylic acid	25.0
Adenylic acid	25.0
Ca pantothenate	0.10
Nicotinamide	0.10
Pyridoxine HCl	2.00
Riboflavin	0.10
Folic acid	0.01
Thiamine HCl	1.00
Protogen or Thioctic acid	1 unit

series of experiments indicate no nutritional differences between the mating types (I, II, III) and strain E insofar as these nutrilites are concerned. In the light of experience with other strains it is unlikely that differences in purine, pyrimidine, and inorganic requirements would be found if sought for.

A comparative growth study of the mating types and strain E in defined medium showed remarkable similarity among the three mating types (Fig. 1). Strain E, perhaps as a result of its long maintenance in test tubes (20 years), grew faster after the third day and reached a higher maximum.

II. General morphology

Mating types I, II, and III have been identified as *Tetrahymena pyriformis* by Corliss (personal communication). There are no apparent morphological differences among the three types. All possess a single macronucleus and at least one micronucleus. The number of micronuclei varies within a single clone; for example, in one series of counts of the Type I clone the number varied as follows: 1 micronucleus, 78%; 2 micronuclei, 16%; 3 micronuclei, 3%; and 4 micronuclei, 2%. A similar range was found in Type II clones. An occasional individual without a micronucleus (about 1%) can be found in laboratory cultures but no amicronucleate clones have been established from nature.

The presence of more than one micronucleus complicates the cytological picture when the steps in conjugation are traced. Since unimicronuclear clones were desirable for cytogenetic studies of conjugation an effort was made to control the number of micronuclei within a clone.

Seventeen clones of mating types I and II were established and after 2–5 days of incubation were stained and the micronuclei counted. One hundred individuals from each clone were examined for their micronuclear number. Of the 17 clones, 7 were found to be as variable as the original stocks; 6 proved to be consistent for bimicronucleate individuals; and 4 contained only unimicronucleate organisms. These 4 clones with single micronuclei were carried through three serial transfers and, after two weeks, stained preparations were made from the last transfers None of these clones had remained constant for single micronuclei as indicated by average counts. However, no exconjugants with more than one micronucleus have been observed and, since multimicronucleate conjugants occur regularly, one of the results of the sexual process is to establish uniformity in the micronuclear number for the species.

The maintenance of a constant number of micronuclei in clone cultures seems to depend on chance alone. The presence of multi-micronucleate cells may be correlated with the formation of doublets (two-mouthed individuals) as suggested by Corliss (personal communication).

The possibility that the number of micronuclei might be influenced in some way by age of the culture was tested by staining 2-, 8-, and 40-day old cultures for examination. Data derived from such preparations gave no indication that the number of micronuclei was correlated with age.

During the first prezygotic division of conjugation, chromosomes (or chromosome aggregates) become clearly visible. There are four (or possibly five) rela-

tively large, thick rods which presumably represent the haploid number. Because these "chromosomes" are comparatively large and so few in number when compared to most other ciliates they provide excellent material for detailed cytological studies.

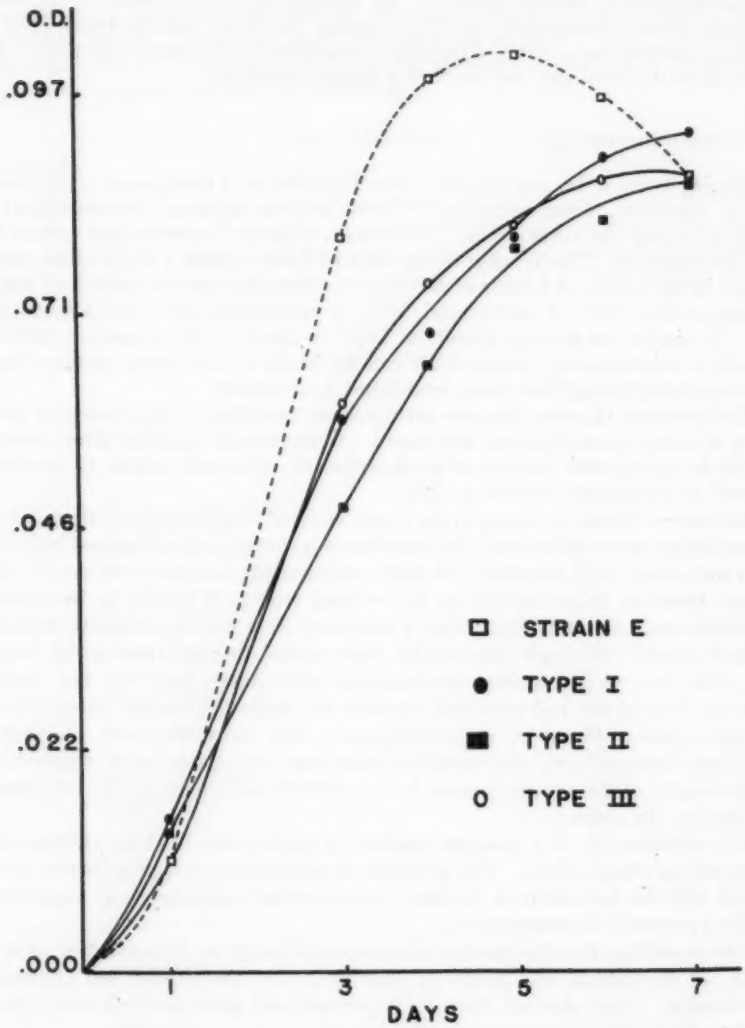


FIGURE 1. Comparative growth of strain E and the three mating types in defined medium. Growth as measured by optical density (O.D.) is plotted against time in days.

III. Conjugation

When washed cells of opposite mating types are mixed under appropriate conditions conjugation takes place. In order to rule out the possibility of selfing, several checks were made. By mixing a small number of cells (15–20) of one mating type with a great many (500 or more) of the opposite mating type and counting the number of pairs that appear as compared to the control in which equal numbers of cells were mixed, it was possible to prove conclusively that selfing did not occur. Furthermore, selfing has never been seen when the mating types are subjected to the same conditions that initiate the process in our selfing (AA) strains.

The preliminary mating reactions reported for some other ciliates, *i.e.*, agglutination and the formation of large clumps of animals, are absent in *Tetrahymena*. Pairs of cells are formed directly. Two animals become attached at their preoral surfaces near their anterior ends with bodies flaring at a wide angle. They are loosely attached at first and often twist and break apart. The attachment soon becomes quite firm. A conjugating pair remains active and swims as a unit with a characteristic spiraling motion.

The following description is intended to cover the normal process of conjugation in *Tetrahymena pyriformis* as seen in mating types I and II. Observations on matings between I and III, and II and III show no differences greater than the normal variation found when mating I and II. The general features of conjugation resemble those reported by Maupas (1889) for *Tetrahymena* [*Leucophrys*] *patula* (Corliss, 1953), and recently by Nanney (1953) for the selfing AA (Ann Arbor) strains of *Tetrahymena pyriformis*. In certain details the phenomenon strikingly resembles the description of autogamy in *Tetrahymena rostrata* as given by Corliss (1952a). Abnormalities, such as the occurrence of multimicronucleate conjugants and triples in conjugation, are ignored in the present paper. Also, no attempt has yet been made to work out the exact time relationships of the sequence of stages in conjugation.

A. Cytology. Cytological studies were made from material fixed in hot Schaudinn's solution and stained according to Dippell and Chao's modification of the DeLameter stain (Sonneborn, 1950). A fast green counterstain was applied in most cases. Fixed material was processed in bulk in centrifuge tubes.

The following stages are indicated diagrammatically in Figure 2. Photomicrographs of some of the stages appear in Plate I. During conjugation two cells of opposite mating types are attached only at their oral surfaces near their anterior ends.

In preconjugants and in recently attached conjugants the single micronucleus is located near the macronucleus (Stage 1). Soon after the conjugants come together the micronucleus elongates into a curved, threadlike "crescent" stage of the first prezygotic division (Stage 2). This crescent later shortens, chromosomes become visible, and the division is completed, resulting in two daughter nuclei in each cell (Stage 3). Each daughter nucleus divides again in the second prezygotic division, producing four nuclei (Stage 4). It is assumed that one of the first two divisions is reductional, as is the case in other ciliates, and that the resulting four nuclei are haploid.

Of the four nuclear products in each conjugant, only one functions in the remainder of the process. The other three eventually degenerate and disappear. The functional haploid nucleus comes in contact with the cell membrane between the conjugants where it undergoes a third prezygotic division to yield two gametic nuclei

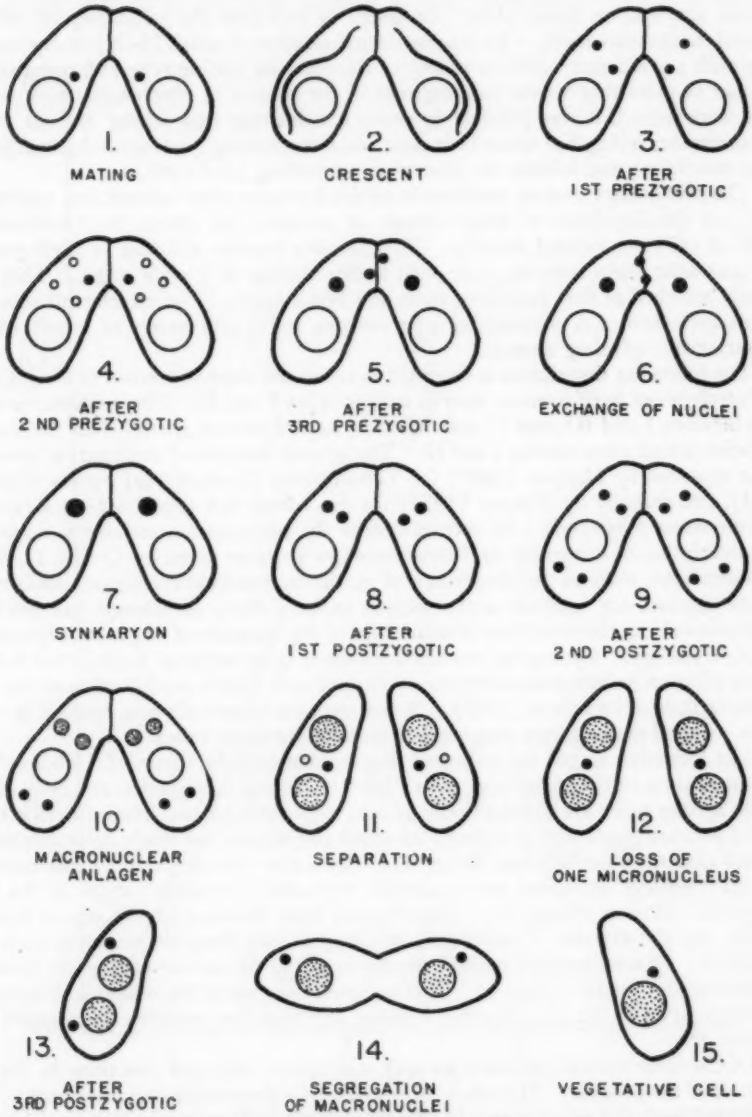


FIGURE 2. Schematic figures showing representative stages in normal conjugation of the WH strains.

(Stage 5). One of these (the stationary nucleus) takes a position anterior to the macronucleus. The other (the migratory nucleus) remains on the membrane.

In the fertilization process the migratory nucleus from each conjugant passes through the cell membrane and fuses with the stationary nucleus of the opposite cell to form the synkaryon (Stages 6 and 7).

The synkaryon then undergoes two postzygotic divisions to produce four daughter nuclei in each conjugant (Stages 8 and 9). Two of these new nuclei are located in the anterior region of the cell, and two in the extreme posterior with the macronucleus lying between. The two anterior nuclei gradually enlarge to become macronuclear anlagen which later develop into two new functional macronuclei (Stage 10). The two posterior nuclei remain small and become new micronuclei.

At about this time the conjugants separate and the old macronucleus in each exconjugant becomes smaller, more intensely stained, and spherical, eventually disappearing without fragmentation. The two anlagen become large new macronuclei and the two new micronuclei usually come to lie in the center of the cell between the macronuclei (Stage 11). One of these micronuclei degenerates, the other undergoes a third postzygotic division (Stages 12 and 13).

Each exconjugant then goes through a single fission, segregating one new micronucleus and one new macronucleus to each daughter cell (Stage 14). This fission completes the sexual process. From the original pair of conjugants four daughter cells have been produced, each of which has been restored to the normal vegetative condition with one macronucleus and one micronucleus (Stage 15).

B. Establishment of exconjugant clones. The usefulness of *Tetrahymena* as a genetic tool requires that the investigator be able to perform easily and dependably the many cross-matings involved in any genetic study with sexually reproducing laboratory animals. He must be able to mate parental types, isolate and culture exconjugant clones (F_1 generations), and then interbreed these to obtain F_2 and succeeding generations. Part of this has already been accomplished and preliminary investigations indicate that other required matings can also be made successfully.

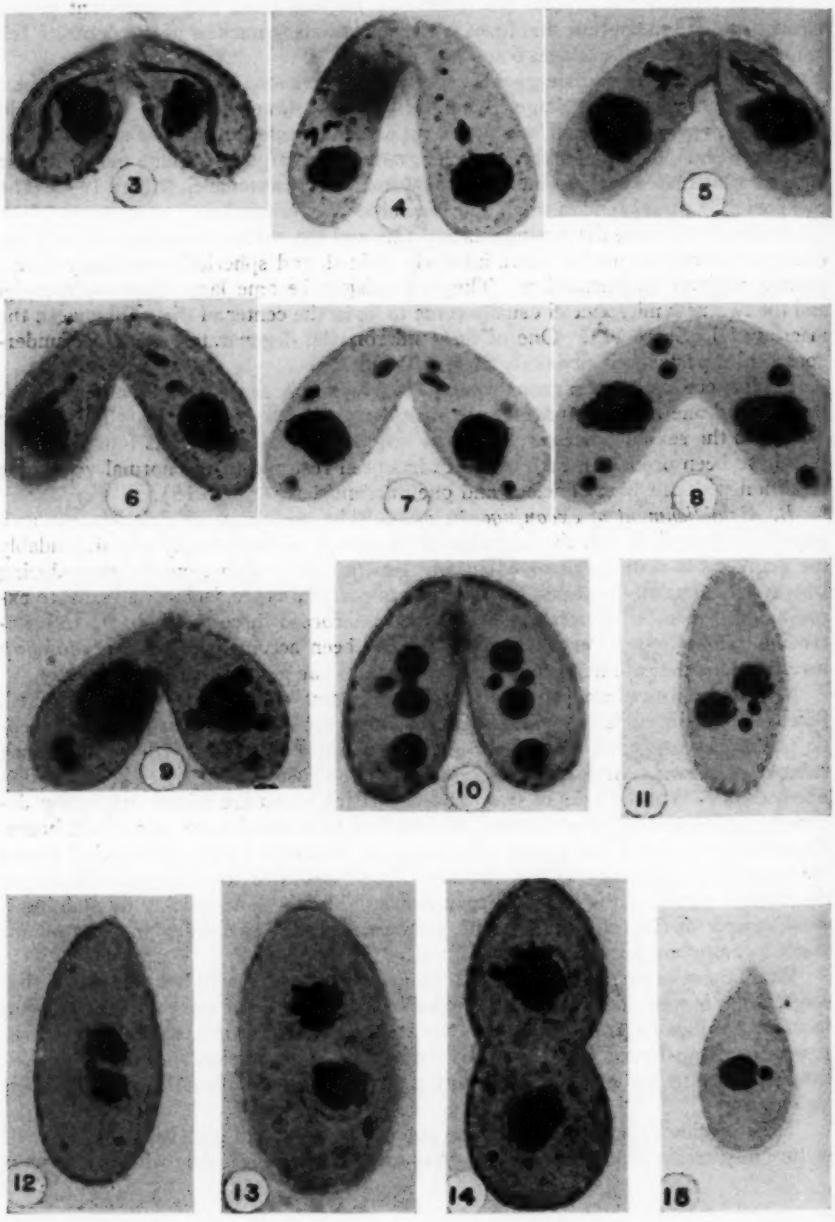
A routine procedure for obtaining exconjugant clones has been established. Since only axenic cultures of *Tetrahymena* are employed, sterile glassware and aseptic techniques are used throughout. Actively growing, young (2–7 day) stock cultures of known mating types are centrifuged and washed four times in sterile distilled water. Washed cells of two opposite mating types are mixed in shallow depression slides and allowed to stand undisturbed in distilled water for 12–24 hours. Examination under a low power microscope at intervals during this period shows the progress of pairing. It has also been found desirable to check periodically on the nuclear status of the conjugating animals. This is done quickly by staining a small sample of the cell mixture with acetocarmine and examining the wet mount under a compound microscope.

When these examinations show that many or most of the cells are paired and that conjugation is well under way, as revealed by the presence of macronuclear anlagen in many conjugants, individual pairs are isolated from the mixture with a micropipette and transferred to drops of distilled water in depression slides. These isolated pairs are then allowed to stand until the conjugants complete the sexual process and separate.

The resulting exconjugants are then picked up singly and transferred to drops of culture medium in depression slides. Within 48 hours each isolate will usually have

PLATE I

Photomicrographs of stages in conjugation of *Tetrahymena pyriformis*, mating types I and II.



divided 4–8 times and the daughter cells are then transferred *en masse* to a tube of medium to establish an exconjugant clone. This clone is maintained thereafter by routine serial transfers at weekly (or longer) intervals.

Mention was made earlier of the desirability of waiting until conjugation was nearly completed before isolating conjugating pairs. It has been found that in most cell mixtures there is a certain amount of abortive conjugation, that is, of individuals coming together but separating after a time without actually conjugating. In some cases this premature separation can probably be blamed on mechanical agitation. Conjugants are only loosely attached at first and if picked up too soon are broken apart in the pipetting process. However, there seem to be other, as yet unknown, factors involved. For no apparent reason some mixtures yield a higher percentage of exconjugants than others. For example, from 154 isolated pairs 26 exconjugant cultures (17%) were obtained. From the separate groups of 20–30 isolates making up the larger series, yields of successful exconjugant cultures varied from 0% to 38%. Some of the failures can definitely be attributed to improper handling such as pipetting too early before conjugants are firmly attached and to a failure to recover isolates. Other failures, however, seem to be due to spontaneous separation of paired individuals.

Because of this high percentage of false conjugation it has been found necessary to verify each "exconjugant" by other tests. One method used is to demonstrate new macronuclei and new micronuclei in one separated individual from each isolated pair. Acetocarmine staining clearly brings out the nuclear picture. However, it is laborious and time-consuming to isolate, stain, and locate on the slide a single organism. An easier test, and one which has proved to be just as valid, is based on the fact that exconjugant clones go through a period of sexual immaturity, that is, a period during which they show no mating reaction (see below). In practice, then,

FIGURE 3. First prezygotic division showing micronuclei elongated into characteristic crescents (Stage 2).

FIGURE 4. First prezygotic division in which the crescents have condensed revealing three distinct "chromosomes." At least one other "chromosome" does not show in this photograph. These are presumably tetrads.

FIGURE 5. A stage slightly later than that shown in Figure 4, in which the "chromosomes" have separated and are moving to the poles in anaphase.

FIGURE 6. After first prezygotic division. One of the nuclei in the right conjugant is entering the second prezygotic division (Stage 3).

FIGURE 7. Four nuclear products of the second prezygotic division. Three relic nuclei are degenerating and one functional nucleus in each conjugant is undergoing the third prezygotic division (Stage 4).

FIGURE 8. Four nuclei resulting from the second postzygotic division can be seen in the left cell (Stage 9). This second division has not yet occurred in the right cell.

FIGURE 9. The two anterior nuclei in the right cell are enlarging to become macronuclear anlagen (Stage 10).

FIGURE 10. A later stage which shows the old macronuclei degenerating.

FIGURE 11. A recently separated exconjugant (Stage 11).

FIGURE 12. A later exconjugant stage in which one micronucleus has disappeared (Stage 12).

FIGURE 13. The functional micronucleus is undergoing the third postzygotic division. The exconjugant is now larger due to feeding.

FIGURE 14. Division of the micronucleus is complete and cytoplasmic cleavage is well underway, segregating a new macronucleus to each daughter cell (Stage 14).

FIGURE 15. Normal vegetative cell (Stage 15).

all newly established clones derived from "exconjugants" are mixed with mating types I, II, and III. If conjugation occurs, the clone is discarded. If conjugation does not take place the clone is considered a true exconjugant.

C. Factors influencing conjugation

The primary requirement for demonstrating conjugation in *Tetrahymena* is, of course, the mixing of organisms of two appropriate mating types. However, there are other factors involved which influence the mating process. As shown in the following experiments the conditions under which the cells are mixed can determine the rate at which conjugation takes place and whether or not it will occur at all.

1. Starvation. Conjugation has never been observed in the presence of nutrients, even in old cultures. However, mating can be regularly initiated by washing, which induces rapid starvation. Cells taken from any phase of the growth cycle, or those that have passed the peak growth (one to four weeks old), and washed in distilled water by centrifugation initiate conjugation at rates which are, within limits, proportional to the number of washings. The period between mixing and the appearance of the first conjugating pairs is here termed the *refractory period,* which is approximately 8 hours at room temperatures (20–22° C.) after washing once in distilled water. The refractory period is decreased to 6 hours when the cells are washed twice. Four washings reduce the time to approximately 4 hours and any more washings fail to decrease this time any further.

Additional evidence that conjugation occurs only between starved animals was obtained in the following way. Washed cells (4 times) were allowed to stand in distilled water for varying lengths of time. These starved cells decreased the refractory period at a rate which depended, within limits, on the length of time they remained in the water before mixing. For example, cells permitted to stand 24 hours in water mated in 1½ to 3 hours whereas those that starved 48 hours mated in 1–2 hours. Apparently, freshly washed cells retain sufficient reserve foods to delay mating.

Direct observation of the protozoa tends to verify this starvation requirement for mating. There is usually a definite and easily recognizable difference in appearance between cells freshly washed and those "ready" to mate. The former are large, vacuolated, and relatively sluggish whereas the starved cells, which are about to conjugate, are smaller, slender, devoid of vacuoles, and quite active.

2. Temperature. Temperature also plays an important part in the mating reaction. Several experiments were performed in an attempt to determine the optimum temperature for conjugation. Depression slides containing mixed washed suspensions of types I and II were incubated at different temperatures and examined at frequent intervals to detect the onset of conjugation. It was found, as shown in Table II, that conjugation began sooner at about 38° C. than at lower temperatures. This effect might be due to increased metabolism at higher temperatures leading to a more rapid depletion of food reserves.

3. Surface area-volume relationships. It has been observed that mating occurs slowly and involves only a few pairs in deep suspensions of washed cells. The same cell mixture will produce abundant conjugating pairs in a much shorter time when placed in shallow containers such as depression slides or watch glasses. The influence of fluid depth on the refractory period is illustrated by the following experiment.

Ten ml. of a distilled water suspension of thoroughly washed cells (types I and II) were placed in test tubes in which the fluid depth was 80 mm. and in small beakers in which the fluid depth was 8 mm. at 22° C. The refractory period was prolonged from 5–7 hours in the beakers to 22–28 hours in the test tubes.

Anoxia is possibly the reason for failure to obtain conjugation in deep containers. Aeration of deep suspensions by bubbling air through the fluid failed to speed up mating but this failure may have been secondarily induced by agitation alone.

4. Agitation. Mechanical agitation (shaking, stirring, etc.) of a mixture, which under quiet conditions mated normally, prevented conjugation completely. Disturbing the protozoa during mating by drawing them into and forcing them out of a pipette also stopped conjugation. It appears that they must remain relatively quiet if mating is to be successful.

5. Age of culture. There are visible differences between young (2–4 day) stock medium cultures and those a week or more old. During the first 4 or 5 days of incubation all organisms in the tube are suspended throughout the medium with heavier concentrations in the upper levels. Longer periods of incubation result in a gradual accumulation in the bottom of the tube of debris composed of cellular fragments and many swollen, distorted and sluggish organisms. After a month there

TABLE II

Effect of temperature on conjugation

Temperature °C.	
1–2°	No conjugation, cells spherical and motionless
23–25°	Conjugation in 6–8 hours
27–28°	Conjugation in 3–5 hours
31–32°	Conjugation in 3–4 hours
37–38°	Conjugation in 3 hours
40–42°	No conjugation
46–49°	No conjugation, cells dead in 2 hours

are very few active "normal" individuals. The question as to whether or not there was any correlation between this change in appearance and activity induced by age, and the refractory period was answered in the following manner. A series of (type I and II) stock medium cultures of varying ages (3, 5, 7, 11, 13, 35 days) was centrifuged and washed through four changes of distilled water. The organisms were then mixed in shallow depression slides and examined at intervals to record the appearance of the first mating pairs. In all cases the activation period was 2–4 hours in length. Age of the culture, within these limits, has no influence on the length of the refractory period.

6. Sexual immaturity. All exconjugant clones of *Tetrahymena* established thus far show a temporary loss of mating type. For a certain length of time after isolation the young clone does not conjugate with other clones. This is believed to be the same period of sexual immaturity reported for some varieties of *Paramecium* and other conjugating ciliates (Sonneborn, 1939).

A total of 52 exconjugants from various matings has been isolated and maintained as clone cultures. All of these failed to conjugate with parental types I, II, or III, or with each other, for two to four weeks after isolation. Serial transfers had no influence on the duration of this period. In several cases, where tested, first trans-

fer cultures regained mating ability at the same time as fourth, fifth and sixth transfers.

It is believed that length of the immaturity period is determined by fission rate, *viz.*, the exconjugant must undergo a certain number of cell divisions before the clone becomes sexually mature as observed by Maupas (1889) in *Tetrahymena patula*. If this is correct, cultural conditions which speed up the fission rate should correspondingly shorten the immaturity period.

Cell division and population growth are influenced within certain limits by temperature. Initial growth is faster at 32° C. than at 22° C. Also, flask cultures with larger surface areas develop heavier populations than tube cultures (Elliott *et al.*, 1952). To determine the effect of faster growth on the time required to reach maturity, parallel series of flasks and tubes of stock medium were inoculated from recently isolated immature exconjugant clones. Sets of cultures were incubated at 22° C., 25° C., and 32° C., and at daily intervals cells were removed, washed, and tested to determine their ability to conjugate with types I, II, and III. At 32° C. both flask and tube cultures did not conjugate until the 18th day after isolation, at 25° C. both flask and tube cultures matured on the 19th day. Those cultures incubated at 22° C. did not mature until the 26th day. Although these results are not conclusive, they indicate that the length of the immaturity period is correlated, in part, with fission rate. Further experiments are planned to demonstrate this correlation more precisely and to develop culture techniques which will shorten the maturation time as much as possible.

Discussion

At intervals during the long period that *Tetrahymena pyriformis*, strain E, has been grown in axenic cultures (since 1932), attempts were made to induce conjugation with other strains with the hope that, should this be possible, its genetic system might be studied. Unfortunately, conjugation was never observed in any of the long established strains (E, W, GL, H) when mixed in all combinations (among themselves and with mating types I, II, and III) and under conditions which normally induce conjugation in other ciliates. Failure may be attributed to the fact that none of them possesses micronuclei. However, amicronucleate ciliates have been known to conjugate. Schwartz (1939) observed mating in *Paramecium bursaria* between experimentally enucleated individuals and normal cells. Diller (1936) reported conjugation between micronucleate and amicronucleate races of *P. aurelia*. From this evidence it seems possible that conjugation can be obtained in some of the many amicronucleate strains of *T. pyriformis* by mixing them with micronucleate strains although this has never been observed in several hundred trials. However, information reported here seems to point to the fact that there exist in nature other mating types and other varieties of *T. pyriformis*. If and when these are found, some of the problems concerning genetics and the significance of amicronucleate races may be solved.

Long ago Maupas (1889) described the cytological details of conjugation in a number of ciliates, among them *"Leucophrys patula,"* a species recently transferred to the genus *Tetrahymena* (Corliss, 1952b, 1953). More recently Horn (1951) observed mating in a strain of *Tetrahymena pyriformis*. Corliss (unpublished work) has studied conjugation in strains of *Tetrahymena* [*Glaucoma*]

parasitica (personal communication). All of these workers were using bacterized cultures and they observed only selfing in which no exconjugants survived. The strains of selfers isolated from the Ann Arbor region (AA strains) mate readily under axenic conditions but they, too, fail to survive the process. The widespread occurrence of a sexual phenomenon so deleterious to the survival of a species as selfing seems to be, at least as it occurs under laboratory conditions, stimulated a search for conjugating strains with surviving progeny. Assuming that conjugation is beneficial to a species it is reasonable to expect that somewhere in nature mating types of *Tetrahymena* exist. The success of this search was enhanced by the fact that the mating types described here do conjugate readily under axenic conditions. Had this not been the case any genetic studies involving nutrition would have been greatly handicapped if not impossible.

It is well known that nutritional conditions of the medium are important in the sexual behavior of ciliates. For example, Chatton and Chatton (1929a) found that *Glaucoma scintillans* underwent conjugation only in the presence of certain bacterial metabolic products while Seckbach (1948) found that bacteria were unnecessary when rye grain, liver, and intestinal mucosa extracts were used. In the present investigation it is possible to control the nutrition in all respects which emphasizes the usefulness of *Tetrahymena* for genetic studies.

The nutrient requirements, insofar as the amino acids and B-vitamins are concerned, are identical for the three mating types and strain E. This is not surprising in view of the remarkable consistency in the requirements of the long established strains. Kidder and Dewey (1945) were able to show some differences in carbohydrate fermentation among several strains of *Tetrahymena pyriformis* (H, E, T-P, T, W, GHH) and *T. vorax* (V_2, PP). With the exception of strains W and GHH, which grew without serine, all required the eleven amino acids that are also essential for the mating types. Likewise, the vitamin requirements of the mating types correlate closely with that of the long established strains. Until more information is available for other closely related ciliates it is impossible to conjecture how universal these requirements are.

The conjugation process in *Tetrahymena* agrees with the generalized sequence of nuclear and cytoplasmic events established for other ciliates. It involves a temporary pairing of two animals, three prezygotic divisions of the micronucleus producing haploid gametic nuclei in each conjugant, mutual exchange of gametic nuclei and their union to form a synkaryon, three postzygotic divisions of the synkaryon, and subsequent reorganization stages of the exconjugants. The most significant difference between the mating types of *Tetrahymena* and the "selfers" described by Maupas (1889), Horn (1951) and Nanney (1953) lies in the fact that viable exconjugant clones are produced. Selfing seems to be a lethal process, whereas exconjugants from the Woods Hole strain undergo a third postzygotic division of the functional micronucleus and divide to produce viable progeny.

In reviewing the literature concerning conjugation and the mating reactions of ciliates one is impressed in many cases with the extreme periodicity or irregularity of the process. Whether or not conjugation takes place when two appropriate cultures are mixed seems to depend on a number of exacting environmental and physiological conditions. The induction of conjugation in *Tetrahymena* seems remarkably easy and simple by contrast.

Wichterman (1953) reported that time of day had a pronounced influence on

the mating reaction in *Paramecium bursaria*. With a few exceptions mating does not occur in the early morning hours or after 5 or 6 P.M., and the reaction is greatest at 12 o'clock noon. Similar diurnal periodicities were found by Jennings (1939) in his varieties of *P. bursaria* and by Sonneborn (1939) in some varieties of *P. aurelia*. Conjugation has been obtained in *Tetrahymena* at any time of day or night. There is no evidence of a diurnal periodicity of any sort, or of sexual inhibition by light or darkness.

The effect of temperature on conjugation has also been investigated by several workers. Sonneborn (1939) found striking varietal differences in this regard with *P. aurelia*. Although mating types of variety 1 will conjugate at any temperature within the range examined, 9° C. to 32° C., mating types of variety 2 will not react above 24° C., and types of variety 3 not above 27° C. Strong mating reactions in *P. caudatum* at temperatures ranging from 18° C. to 24° C. were reported by Gilman (1939). Giese (1939) in a study of temperature effects on conjugation in *P. multimicronucleatum* observed that animals grown at 30° C. seldom were found to mate but did so when placed at lower temperatures. *Tetrahymena* conjugates readily at all temperatures within the range 17° C. to 38° C.; temperatures below 17° C. have not been investigated thoroughly. The principal influence of temperature seems to be on the "refractory period." Mating takes place sooner at 38° C. than at lower ranges.

There are scattered reports in the literature of the influence of certain chemical factors and pH on conjugation in ciliates. For example Sonneborn (1939) reported that the mating reaction in *P. aurelia* is weak or lacking when "deleterious bacteria," or presumably, metabolic products of these bacteria, injure the paramecium. Chatton and Chatton (1929a) studied a variety of chemical compounds, including $CaCl_2$, $FeCl_3$, pyruvic acid, glucose, and bacterial metabolic products as "zygogenic agents" influential in initiating conjugation of *Glaucoma scintillans*. The zygogenic effects of these compounds were re-investigated by Seckbach (1948) who added salts of barium and magnesium to the list of factors essential or stimulatory for conjugation in this species. Wichterman (1953) found that pH within the range 6.0 to 8.0 had little influence on conjugation of *P. multimicronucleatum*. Since we have been able to obtain conjugation so readily in *Tetrahymena* simply by mixing washed mating types in distilled water a search for specific chemical factors has not been made. The pH of unbuffered water suspensions in which conjugating pairs are found varies from 6.6 to 7.7.

The nutritive state of the protozoan seems to be an important factor influencing conjugation in all ciliates. Maupas (1889) lists hunger, sexual maturity, and diverse ancestry as the three conditions necessary for mating in ciliates. Sonneborn (1939) observed that in *P. aurelia* the mating reaction does not take place in cultures that are either over-fed or completely starved. A similar situation exists in *P. caudatum* (Gilman, 1939) and *P. bursaria* and *P. calkinsi* (Wichterman, 1953). Giese (1939) found that food appeared to be the most important single factor in regard to conjugation in *P. multimicronucleatum* and that a decline in available food after a period of plenty was required. Chatton and Chatton (1929b) stated that starvation was a necessary condition for conjugation of *Glaucoma scintillans*. Evidence obtained thus far on the effect of starvation on conjugation of *Tetrahymena* agrees in general with that reported for other ciliates.

Tetrahymena must be at least partially starved before it will conjugate. Unlike those ciliates which seem to require a nutritional state intermediate between well-fed and completely starved, *Tetrahymena* has been found to conjugate normally even after starving in distilled water for 3 or 4 days.

One of the three conditions necessary for conjugation in ciliates, as cited by Maupas, is sexual maturity. Observations by many investigators indicate that in some species, at least, conjugation can be induced only when the animals are sexually mature. According to Calkins (1933) *Uroleptus mobilis* will mate only after a period of from 5 to 10 days following fertilization. Many races of *P. aurelia* (Sonneborn, 1939) do not conjugate during the first week or two; other races, however, lack this period of immaturity and are able to mate again immediately following conjugation. Jennings (1939) reported a state of sexual immaturity for *P. bursaria* ranging from a few weeks to several months. Periods of immaturity have been found in *P. caudatum* by Gilman (1939) and in *Euplotes* by Kimball (1939). *Tetrahymena* also goes through a definite period of immaturity during which exconjugants are not sexually reactive for a week or more. It may be possible to correlate this period with number of fissions.

From the observations reported here it seems quite clear that *Tetrahymena* has potentialities as a tool for further investigations in protozoan genetics and should supplement the already voluminous literature on *Paramecium* and other ciliates. Moreover, because its biochemistry and physiology are well known, it should become an important addition to the list of microorganisms that have already greatly enhanced our knowledge of biochemical genetics.

Summary

1. Conjugating strains representing three different mating types of *Tetrahymena pyriformis* have been isolated from fresh water ponds in the Woods Hole, Massachusetts, area and established in axenic cultures.

2. These strains have the same amino acid and vitamin requirements as *T. pyriformis E.*

3. The cytology of conjugation is described.

4. Routine laboratory procedures for obtaining conjugation and establishing exconjugant clones (F_1 and successive generations) are presented.

5. Some of the factors influencing conjugation in *T. pyriformis* (nutritive state, temperature, oxygenation, agitation, culture age, and sexual maturity) are discussed and compared with reports in the literature dealing with conjugation in other ciliates.

LITERATURE CITED

Calkins G. N., 1933. The biology of the protozoa. Lea and Febiger, Philadelphia.

Chatton, E., and M. Chatton, 1929a. Les conditions de la conjugaison du *Glaucoma scintillans* en cultures léthobactériennes. Action directe et spécifique de certains agents zygogenes. *C. R. Acad. Sci. Paris,* **188**: 1315–1317.

Chatton, E., and M. Chatton, 1929b. L'état de jeûne, condition nécessaire, mais non suffisante, de la conjugaison expérimentale de l'infusoire *Glaucome scintillans. C. R. Acad. Sci. Paris,* **189**: 59–61.

Corliss, J. O., 1952a. Le cycle autogamique de *Tetrahymena rostrata. C. R. Acad. Sci. Paris,* **235**: 399–402.

Corliss, J. O., 1952b. Review of the genus *Tetrahymena. Proc. Soc. Protozool.,* **3**: 3.

CORLISS, J. O., 1953. Comparative studies on holotrichous ciliates in the *Colpidium-Glaucoma-Leucophrys-Tetrahymena* group. II. Morphology, life cycles, and systematic status of strains in pure culture. *Parasitology*, **43**: 49–87.

DILLER, W. F., 1936. Nuclear reorganization processes in *Paramecium aurelia*, with descriptions of autogamy and "hemixis." *J. Morph.*, **59**: 11–67.

ELLIOTT, A. M., 1949. The amino acid requirements of *Tetrahymena geleii* (E). *Physiol. Zool.*, **22**: 337–345.

ELLIOTT, A. M., 1950. The growth-factor requirements of *Tetrahymena geleii* E. *Physiol. Zool.*, **23**: 85–91.

ELLIOTT, A. M., J. F. HOGG, J. V. SLATER AND C. WU, 1952. Improved culture conditions for *Tetrahymena*. *Biol. Bull.*, **103**: 301.

ELLIOTT, A. M., AND D. F. GRUCHY, 1952. The occurrence of mating types in *Tetrahymena*. *Biol. Bull.*, **103**: 301.

ELLIOTT, A. M., AND D. L. NANNEY, 1952. Conjugation in *Tetrahymena*. *Science*, **116**: 33–34.

GIESE, A. C., 1939. Studies on conjugation in *Paramecium multimicronucleatum*. *Amer. Nat.*, **73**: 432–444.

GILMAN, L. C., 1939. Mating types in *Paramecium caudatum*. *Amer. Nat.*, **73**: 445–450.

HORN, GERTRUDE, 1951. Conjugation in *Tetrahymena geleii S*. M. S. thesis. University of Washington.

JENNINGS, H. S., 1939. Genetics of *Paramecium bursaria*. I. Mating types and groups, their interrelations and distribution; mating behavior and self sterility. *Genetics*, **24**: 202–233.

KIDDER, G. W., AND V. C. DEWEY, 1945. Studies on the biochemistry of *Tetrahymena*. III. Strain differences. *Physiol. Zool.*, **18**: 136–157.

KIMBALL, R. F., 1939. Mating types in *Euplotes*. *Amer. Nat.*, **73**: 451–456.

MAUPAS, E., 1889. Le rajeunissement karyogamique chez les ciliés. *Arch. Zool. exp. et gen.*, **7**: 149–517.

NANNEY, D. L., 1953. Nucleo-cytoplasmic interaction during conjugation in *Tetrahymena*. *Biol. Bull.*, **105**: 133–148.

SCHWARTZ, V., 1939. Konjugation micronucleusloser Paramaecien. *Naturwiss.*, **27**: 724.

SECKBACH, MANFRED, 1948. Zygogenic and azygogenic substances for the ciliate, *Glaucoma scintillans*. *Ann. Inst. Pasteur.*, **75**: 156–162.

SONNEBORN, T. M., 1939. Sexuality and related problems in *Paramecium*. *Coll. Net.*, **14**: 77–84.

SONNEBORN, T. M., 1950. Methods in the general biology and genetics of *Paramecium aurelia*. *J. Exp. Zool.*, **113**: 87–147.

WICHTERMAN, RALPH, 1953. The biology of Paramecium. The Blakiston Co., New York.

THE RELATION OF BODY WEIGHT TO BODY SURFACE AREA IN MARINE FISHES

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In the course of attempting to establish a satisfactory basis for comparison of gill areas of certain marine fishes, considerable information about the body surface area has accumulated. Much has been written on the value of knowing the area of the body surface in metabolism studies and the difficulties in obtaining it. The formula most commonly used for determining surface area when the weight is known is $S = K w^{2/3}$, where S represents the area of surface, w is the body weight, and K is a constant for the particular species. Reported values for K have varied from less than 5 to more than 18 for different species. Benedict (1938), who has carefully reviewed at length his own work and that of others as it relates to metabolism and body surface, believes that with the exception of animals that tend to become spherical or greatly elongated, the value of K is in the neighborhood of 10. He gives 10 as "the best value of K" for 21 of 30 birds and mammals, with a range 9 (mouse, guinea pig) to 11.8 (macaque). He further points out, however, that there have been wide discrepancies in the determination of body surface area, even for the same species. More recently Zeuthen (1947) and Kleiber (1947) have also reviewed the subject and have called attention to variations and discrepancies reported in the literature. Kleiber (1947) recommends the use of the ¾ power of the weight as representative of metabolic size.

It is not the purpose here to enter into a discussion of metabolic rate, but rather to make available data concerning the relation of body weight to body surface in certain fishes.

This work was carried out at the Marine Biological Laboratory, Woods Hole, Mass., and at the Duke University Marine Laboratory, Beaufort, N. C., and was supported in part by the Duke University Research Council.

Methods of Obtaining Body Surface Area

A few trials quickly demonstrated that the use of 10 as a value for K in the formula $S = K w^{2/3}$, while giving approximately accurate determinations of body surface area in some fishes, produced wide deviations from experimentally obtained surface areas in other species. Measurements of body surface area were made directly on over 300 individual fish representing 39 species and 25 families. From these observations average values of K for the different species were determined.

Most data were obtained by following the method of Riess (1881), who determined the surface area of pike by drawing around the body, allowing for depth and omitting fins, and then calculating the area of the outline drawing. This procedure proved quite satisfactory for most of the fishes studied and particularly so for the greatly depressed or compressed species, such as flounders, butterfish, and cutlass fish. That greater difficulty was encountered in employing the method on odd-shaped

fishes like the puffer, sea robin, and toadfish is reflected in the wider variations of individual determinations on these species.

A few attempts were made to determine the area of the removed integument of puffers but the skin stretched so readily that it was difficult to obtain consistent results and this method was abandoned.

Until some degree of proficiency was obtained in estimating the depth to allow in making outline drawings of the body of odd shaped fishes, the area of the outlines was checked by the more tedious and laborious method of covering the body with pieces of paper and obtaining the total area of the various pieces. The area of all outlines was secured by use of a planimeter.

Results and Discussion

The values of K in the formula $S = K w^{2/3}$ for 39 species of fish are shown in Table I. It can be clearly seen that if the formula is to be used for the determination of body surface area in fishes, the value of K must be established for each species, or at least for each type of body plan. The more or less rotund species have low values for K while the greatly depressed and compressed species have high values.

Table I

Values of the constant K for marine fishes in the formula $S = Kw^{\frac{2}{3}}$, where S represents the area of body surface and w is the body weight

Family	Species of fish	No. of determinations	Weight in grams			Value of K		
			Max.	Min.	Ave.	Max.	Min.	Ave.
Clupeidae	Menhaden, *Brevoortia tyrannus*, adults	36	729	382	553	8.3	7.1	7.8
Clupeidae	Menhaden, small	10			19			8.7
Clupeidae	Thread herring, *Opisthonema oglinum*, small	4	46	40	43			8.7
Anguillidae	Common eel, *Anguilla rostrata*	4	562	94	345	11.8	10.7	11.3
Leptocephalidae	Conger eel, *Leptocephalus conger*	1	3519	3519	3519	10.2	10.2	10.2
Pleuronectidae	Fluke, *Paralichthys dentatus*	7	524	86	401	12.2	11.6	11.8
Pleuronectidae	Sand flounder, *Lophopsetta maculata*	1	395	395	395	15.3	15.3	15.3
Pleuronectidae	Winter flounder, *Pseudopleuronectes americanus*	2	745	723	734	14.7	14.6	14.6
Trichiuridae	Cutlass fish, *Trichiurus lepturus*	12	131	47	74	13.0	12.2	12.7
Scombridae	Common mackerel, *Scomber scombrus*	23	226	86	155	7.6	6.7	7.2
Scombridae	Bonito, *Sarda sarda*	2	2387	1998	2192	7.0	6.4	6.7
Scombridae	False albacore, *Gymnosarda alleterata*	1	2079	2079	2079	6.9	6.9	6.9
Scombridae	Spanish mackerel, *Scomberomorus maculatus*	9	483	39	234	8.9	7.6	8.3
Mugilidae	Jumping mullet, *Mugil cephalus*	5	250	132	165	8.8	7.5	8.0
Stromateidae	Harvest fish, *Peprilus alepidatus*	11	77	14	24	9.2	8.4	9.1
Stromateidae	Butterfish, *Poronotus triacanthus*	25	261	23	112	9.2	8.1	8.5
Carangidae	Big-eyed scad, *Selar crumenophthalmus*	3	55	52	53	7.2	6.7	7.0
Carangidae	Crevalle, *Caranx hippos*	10	155	80	120	8.8	7.6	8.0
Carangidae	Hard-tail, *Caranx crysos*	3	297	271	287	7.7	7.2	7.4
Centrolophidae	Rudderfish, *Palinurichthys perciformis*	6	294	106	199	7.8	7.1	7.5
Pomatomidae	Bluefish, *Pomatomus saltatrix*	4	1035	184	417	7.8	7.8	7.8
Coryphaenidae	Dolphin, *Coryphaena hippurus*, male	3	7033	2268	4258	9.7	9.2	9.4
Coryphaenidae	Dolphin, female	2	3289	165	1727	8.9	8.7	8.8
Moronidae	Striped bass, *Roccus lineatus*	4	2482	917	1648	8.8	7.5	8.0
Serranidae	Sea bass, *Centropristis striatus*	3	287	230	257	8.3	7.7	8.0
Pomadasidae	Pig fish, *Orthopristis chrysopterus*	1	60	60	60	7.9	7.9	7.9
Sparidae	Scup, *Stenotomus chrysops*	13	581	185	361	8.2	7.0	7.7
Sparidae	Pinfish, *Lagodon rhomboides*	4	58	49	53	8.0	7.0	7.5
Sparidae	Sheepshead, *Archosargus probatocephalus*	15	5216	54	1021	10.0	6.7	8.1
Scienidae	Spot, *Leiostomus xanthurus*	2	111	53	82	8.2	7.7	8.0
Scienidae	Croaker, *Micropogon undulatus*	4	62	40	48	8.1	8.0	8.0
Scienidae	Silver perch, *Bairdiella chrysura*	2	86	40	63	7.8	7.6	7.7
Otolithidae	Sea trout, *Cynoscion regalis*	6	1055	705	807	9.5	7.8	8.6
Ephippidae	Spadefish, *Chaetodipterus faber*	3	400	125	239	8.3	8.0	8.1
Triglidae	Brown sea robin, *Prionotus carolinus*	4	337	155	221	9.3	6.8	8.3
Triglidae	Red sea robin, *Prionotus strigatus*	10	1178	146	365	11.4	6.5	8.2
Labridae	Tautog, *Tautoga onitis*	6	1025	412	580	7.9	7.0	7.6
Tetraodontidae	Puffer, *Spheroides maculatus*	17	440	132	257	9.0	6.8	7.8
Diodontidae	Bur fish, *Chilomycterus schoepfi*	1	313	313	313	6.3	6.3	6.3
Batrachoididae	Toadfish, *Opsanus tau*	58	776	15	233	11.5	6.3	8.9
Lophidae	Goosefish, *Lophius piscatorius*	2	4583	518	2551	9.5	8.8	9.2

Fusiform and moderately compressed species are intermediate in position. Of the species listed in Table I it is perhaps to be expected that the bur fish would have the lowest value for K, for this is a semi-rotund, short-bodied species. Highest values of K were recorded for the greatly depressed flounders. The value of K bears a relationship to habitat and taxonomic position only insofar as shape and relative weight are correlated with habitat and taxonomic position. The K value of fast-swimming pelagic fishes would not be at either extreme, for fast swimmers carry neither excess weight nor excess surface, as is often tolerated by bottom dwelling species.

It has been suggested that a puffer, when swelled to the maximum and thus practically spherical, would have the minimum value for K. This might be true if the fish filled up with water, but of course would not be true if puffed with air, for in the latter case the body surface would be increased without materially increasing the body weight. As a matter of curiosity an attempt was made to determine the surface area of a puffer both before and after swelling. As nearly as could be determined the area of the puffed surface was approximately double that of the unpuffed condition.

None of the fish used was taken during the breeding season and consequently the extra weight of ripe gonads was not a factor. With the exception of the dolphin no noticeable differences were detected between males and females. Dolphins exhibit marked sexual dimorphism and in this case males showed a significantly higher value for K. In other species males and females were lumped together.

Typical curves showing the relation of body surface area to body weight are presented in Figures 1 and 2 for the butterfish, mackerel, menhaden and toadfish. Comparison of the observed body surface area curves with area curves calculated on the basis $K = 10$ shows graphically that it is misleading to assume that the value of K is the same for all species. This is especially apparent for the larger mackerel (Fig. 1) and menhaden (Fig. 2), but is much less obvious for the toadfish (Fig. 2) where the observed value of K (8.9) more nearly approaches 10. There is also the possibility that the value of K is not the same for a species at all ages. Ten young menhaden averaging 19.3 grams in weight had a mean K value of 8.7 while for adult menhaden, weighing from 450 to 650 grams, the mean value for K was 7.8. The young menhaden exceeded the maximum value of K for adults. There is the pos-

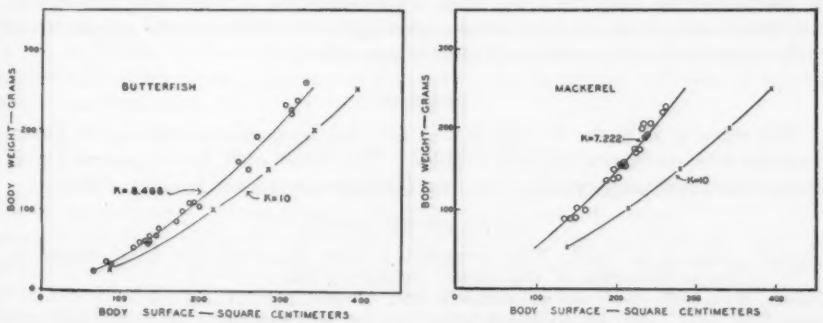

FIGURE 1. Relation of body surface area to body weight in butterfish (*Poronotus triacanthus*) and mackerel (*Scomber scombrus*). For comparison with observed values area computed on the basis $K = 10$ is also shown.

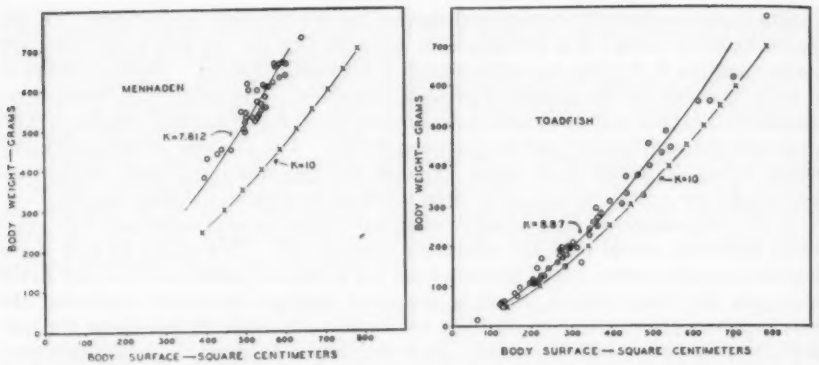

FIGURE 2. Relation of body surface area to body weight in menhaden (*Brevoortia tyrannus*) and toadfish (*Opsanus tau*). For comparison with observed values area computed on the basis $K = 10$ is also shown. The curves of Figure 2 are not on the same scale as those of Figure 1.

sibility in this case that the difference may be accounted for by error in the technique of determining the surface area of the small fish; but it seems more probable to assume that the difference in K value is due to differences in body proportions. Young menhaden are shorter bodied in relation to depth and are more compressed than adults. Four small thread herring of approximately the same shape as the small menhaden also showed higher values for K than did the adult menhaden.

In the surface area determinations on fishes the fins were excluded. Fish fins are modified in many ways and were they included as part of the body surface, the body area determinations would be misleading. For example, it was found experimentally that if the surface area of the pectoral fins of the red-winged sea robin was included as part of the body surface area, the value of K would rise to 17 instead of 8.2 when fins are omitted.

The magnitude of the deviations from the mean in the toadfish (Fig. 2) indicates the difficulty in accurately determining the surface area of some species. This raises the question of the value, in many cases of marine fishes, of expressing metabolism in terms of surface area as opposed to expressing it in terms of body weight, which can be so much more accurately and quickly determined.

SUMMARY

The value of K in the formula $S = K w^{2/3}$ has been determined for 39 species of marine teleosts representing 25 families. The values of K are in general higher for depressed and compressed species than for more streamlined fusiform fishes.

LITERATURE CITED

BENEDICT, FRANCIS G., 1938. Vital energetics, a study in comparative basal metabolism. Carnegie Institution of Washington, Publication, 503.

KLEIBER, MAX, 1947. Body size and metabolic rate. *Physiol. Rev.*, **27**: 511–547.

RIESS, J. A., 1881. Der Bau der Kiemenblätter bei den Knochenfischen. *Arch. f. Naturgesch.*, **47**: 518–550.

ZEUTHEN, ERIK, 1947. Body size and metabolic rate in the animal kingdom with special regard to the marine microfauna. *C. R. Lab. Carlsberg (Chemical Series)*, **26**: 17–161.

A STUDY OF ISOTONIC SOLUTIONS FOR THE ERYTHROCYTES OF SOME MARINE TELEOSTS AND ELASMOBRANCHS

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The assumption is commonly encountered that in all teleosts the osmotic pressure of the blood is equivalent to about 0.25 molar (*M*) NaCl and in all elasmobranchs to about 0.5 *M* NaCl (*cf.*, Baldwin, 1949; Davson, 1951; Prosser *et al.*, 1950; Heilbrunn, 1952). The evidence for these values was obtained by the determination of the bloods' freezing point depression, from which the osmotic pressure was calculated (Botazzi, 1897; Fredericq, 1901; Garrey, 1905; Rodier, 1899; Schlieper, 1930; Smith, 1932; Krogh, 1939). The number of species examined in each class was generally small and no special significance was attributed to the observed variations in blood osmotic pressure between different species.

In addition, few attempts have been made to develop solutions for fish tissues which were balanced with respect to both ion content and concentration. Those of Baglioni (1906) and Fry (1909) were based upon the urea analyses of elasmobranch blood by Schraeder (1890); that of Lutz (1930) upon the data of Smith (1929). The Baglioni and Lutz solutions were used in studies on the elasmobranch heart, while Fry's was used in a study of blood coagulation. The "Manual of Formulae and Methods (III)" of the Marine Biological Laboratory, Woods Hole, lists a few artificial solutions supposedly "physiological" for fish tissues. Our findings reported below indicate that these solutions are not isotonic for red cells of the species studied. Apparently no systematic studies have been undertaken to determine isotonic solutions for fish erythrocytes. Solutions calculated from ion analyses of body fluids, such as those of Smith (1929) cannot, without experimental study, be taken as isotonic for red cells.

We have found that a solution of NaCl which was isosmotic with a sample of fish serum, as determined by the depression of the freezing point, was hypertonic to the red cells of that fish. This general finding led us to investigate other methods for determining solutions which were isotonic for the red cells of teleost and elasmobranch bloods.

Materials and Methods

Blood was collected from the elasmobranchs by syringe from either the caudal vessels or the heart. In the teleosts blood was taken from the heart. In the early work, blood from mackerel and tautog was obtained from the severed branchial arteries. When a sample of blood from a single fish was less than 10 ml., blood from several fish of the same species was usually pooled for study. The blood was defibrinated by gentle stirring with a rubber-tipped glass rod. In a few cases sodium oxalate was used as an anticoagulent instead of defibrination. The blood was filtered through gauze to remove small clots and, if not immediately used, was

stored at 7° C. All measurements were made at about 23° C. with blood that was less than eight hours old. Blood was collected from all of the fish immediately after their removal from sea water. In most cases the fish were caught in a fish weir.

Four separate experimental approaches were used to investigate either cell volume changes or the osmotic concentration of the serum. These four were: (1) cryoscopy, (2) hematocrit, (3) hemoglobin concentration and (4) densimetry.

Cryoscopy, the only method which does not measure cell volume, was used to determine the osmotic concentration of serum and of equivalent NaCl solutions. This method is only useful in determining isosmotic solutions, not isotonic solutions.

Measurements of cell volume by hematocrit, hemoglobin concentration and densimetric methods were based on the assumption that cells are at osmotic equilibrium with their environment, *viz.*, serum. In addition these three methods depended upon the cells behaving as osmometers (*cf.* Lucke and McCutcheon, 1932). Confirmatory evidence for this idea is found in our experiment where the relative volume of fish red cells plotted against the reciprocal of the saline concentration of the suspension medium gave a linear relation. The concentration of NaCl in which the cells maintained the same volume as in serum was taken as isotonic.

TABLE I

Freezing point depressions of NaCl solutions

Solution of NaCl in moles per liter	ΔT_f
0.05	−0.197
0.10	−0.372
0.15	−0.576
0.20	−0.771
0.25	−0.943
0.30	−1.133
0.35	−1.328
0.40	−1.449
0.50	−1.816
0.75	−2.690
1.00	−3.692

The freezing point depression (ΔT_f) of serum or whole blood was measured with the standard Beckman apparatus by the method of Findlay (1941). The ΔT_f of a series of NaCl solutions of known molarity was also determined by the same method. The NaCl used in preparing these solutions was Merck reagent grade sodium chloride "for biological work" (Williams and Jacobs, 1931). From these data a calibration curve of ΔT_f versus known *molarities* of NaCl was prepared, and isosmotic solution of NaCl interpolated from this curve from the ΔT_f measurements of fish sera. Table I presents the data from which this curve was constructed. Our NaCl solutions were unbuffered and well-aerated.

The hematocrit method of Parpart and Ballentine (1943) was used to compare the volume occupied by a known quantity of red cells in a NaCl solution of known concentration. By the use of this method one was able to determine the particular NaCl solution in which the cells occupied the same volume as in serum.

Since the loss of hemoglobin from red cells is considered an all-or-none phenomenon (Parpart, 1931), the amount of hemoglobin in a given volume of cells

can be used as a measure of the quantity, *i.e.*, volume, of red cells present in a suspension (Parpart and Green, 1951). In practice, red cells in either serum or NaCl solutions were packed at 15,000 G for 10 minutes. Twenty cmm. of packed cells were hemolyzed in a given volume of 0.03 *M* NaCl containing 0.003 *M* phosphate buffer at pH 7.4. The concentration of hemoglobin was measured spectrophotometrically at 540 mμ. NaCl solutions which consistently maintained red cells at a volume, such that the hemoglobin concentration of packed samples was equal to the hemoglobin concentration of packed samples of cells in serum, were considered isotonic with serum.

The densimeter technique of Parpart (1935) was used to record rapid volume changes in red cells. This technique, which automatically records the amount of light transmitted through a red cell suspension, is capable of following volume changes of cells exposed to suspension medium from the first second onwards. By the use of the densimeter, NaCl solutions were found in which the volume of the cells was maintained constant and equal to that in serum. Since this instrument permitted the rapid determination of isotonic NaCl solutions, it was used chiefly in survey studies of a number of fish bloods.

TABLE II

Molar values of isotonic NaCl solutions
Species of fish

Method of determination	Mackerel			Tautog			Smooth dogfish					
	No. of fish	No. of dtm.	Value moles NaCl	No. of fish	No. of dtm.	Value moles NaCl	No. of fish	No. of dtm.	Value moles NaCl	No. of fish	No. of dtm.	Value moles NaCl-Urea†
Hematocrit	67	10	0.27±.007	21	7	0.21±.007	11	5	0.34±.008	14	7	0.47±.004
Hemoglobin	41	6	0.26±.005	6	3	ca. 0.23	10	4	0.35±.02	12	5	0.51±.009
Densimeter	67	10	0.26±.004	23	8	0.26±.007	13	6	ca. 0.53	14	7	0.52±.005
ΔT_f*	59	9	0.30±.004	23	8	0.27±.008	13	6	0.53±.003	13	6	0.53±.003

* Isosmotic solutions.
† Equal molecular amounts of NaCl and urea expressed as equivalent moles of NaCl.

RESULTS AND DISCUSSION

Table II presents values of isotonic solutions of NaCl for the red blood cells of mackerel, tautog and dogfish obtained by three of these methods. Also included in this table are isosmotic values for these cells as determined by ΔT_f measurements of their sera. The measure of variation is indicated by the standard error.

No significant differences in tonicity of NaCl solutions for a given species were obtained by the use of hematocrit and hemoglobin methods. Both of these methods measured the equilibrium volume attained by the cells after one hour of exposure to serum or NaCl solutions. Of the three species studied by these two methods, each would appear to require isotonic NaCl solutions of differing molarity. Statistically the differences between 0.27 *M* for mackerel, 0.21 *M* for tautog and 0.34 *M* NaCl for dogfish are real.

The densimeter measurements of isotonicity for these three species are not in

complete agreement with those obtained by hematocrit and hemoglobin methods. The most unsatisfactory measurement is that for tautog blood. We do not know why this value is high or, perhaps, why the hematocrit-hemoglobin values are low. If the hematocrit and hemoglobin values are low, the cells may have decreased their internal osmotic pressure. This is possible because these methods require a longer time to determine the isotonicity (between one and two hours) than does the densimeter method, which makes such measurements within a few minutes.

When dogfish red cells are taken from serum and placed in a NaCl solution which does not contain urea, urea diffuses from the cells and within nine minutes a new equilibrium volume is reached. But when the initial volume of dogfish red cells is obtained by use of the densimeter, by using only the first 10 seconds of exposure, it is found that a NaCl solution of 0.53 *M* is "isotonic." This is because no water exchange is possible now and the only volume change which will be observed will be shrinking as urea diffuses out of the cells. This volume change due to the outward diffusion of urea was not noticeable under our conditions of measurement until about 30 seconds after the cells were exposed to the 0.53 *M* NaCl solution. Thus the apparent discrepancy in isotonic volumes for dogfish red cells between densimeter and hematocrit measurements reflects the loss of urea in the latter. The urea plays no role in determining the final equilibrium volume of these cells as was shown by Green and Hoffman (1951).

The NaCl-urea solution indicated in Table II contained both urea and NaCl in 0.35 *M* concentrations. Such a mixture is equivalent osmotically to 0.53 *M* NaCl. Aqueous dilutions of this NaCl solution expressed as the equivalent moles of NaCl appear in Table II as isotonic solutions for dogfish red cells. The isotonic value of 0.47 *M* obtained by the hematocrit method is significantly different from that arrived at by other methods. This variance is not understood. The value of using NaCl-urea solutions is to prevent urea-containing cells from undergoing any volume change. The results shown in Table II indicate that the NaCl-urea solution, containing 0.35 *M* of each of these reagents, is approximately isotonic.

The column headed ΔT_f in Table II lists the isosmotic values in moles of NaCl of the sera of these three species of fish. In general these isosmotic values are higher than the isotonic values obtained by the other methods. In the case of mackerel serum this difference is statistically significant. The reason for this hypertonicity of isosmotic solutions may be attributed in part to the difference in the activity of ions at the freezing point and at room temperature. Of more importance is the response of the cell to a solution containing a single species of cation. In such a solution the cell surface may be modified or the rate of water penetration may be different from that in plasma. Either of these conditions could account for this hypertonicity.

Table III shows the actual freezing point depressions of the bloods of the fish in Table II. The percentage of cells of the teleosts was determined in defibrinated blood obtained from cutting the gills. These are somewhat lower than values found for blood obtained from heart puncture. The pH values are for aerated blood and quite similar to the pH of the blood of other vertebrates.

The data in Tables IV and V were obtained using the densimeter method for the determination of isotonicity. The accuracy of this method is $\pm$ 0.001 *M* of NaCl and the maximum variation (for blood from the heart or caudal vessels) among fish of the same species was $\pm$ 0.01 *M* NaCl.

TABLE III

Physical data on fish bloods

Kind of fish	No. sampled	No. of measurements	pH		% cells	Freezing point °C.
			Average	Range		
Mackerel	57	8	7.40	7.31–7.57	21.2±0.4*	−1.13
Tautog	17	5	7.27	7.08–7.48	20.5±0.8	−1.05
Dogfish	14	7	7.50	7.35–7.83	20.6±0.07	−1.95

* Standard error.

Table IV shows the dependence of the isotonic value upon the method of obtaining the sample of blood. The results with heart blood are thought to be closer to the true tonicity since the gill blood is probably contaminated with sea water and body fluids. Very striking is the fact that for either heart or gill blood there is a large variation in values between the various species of teleosts. The range is 0.10 *M* NaCl for the shark sucker to 0.26 *M* NaCl for the mackerel. These data necessitate a revision of the apparently over-generalized concept that the isotonic values for the teleosts fluctuate slightly around 0.25 *M* in NaCl, and also of our ideas concerning water balance in these forms.

Table V lists the isotonic values obtained for different elasmobranch bloods *after* osmotic equilibrium was established between the cells and the NaCl solution. In addition certain solutions listed in the "Formulae and Methods III" of the Marine

TABLE IV

Molar values of isotonic solutions for teleost red cells

Name of fish	No. studied	No. detn.	Average isotonicity Moles of NaCl	
			Blood drawn from	
			Gills	Heart
Shark sucker (*Echeneis naucrates*)	2	2	—	0.10
Weak fish (*Cynoscion regalis*)	1	1	—	0.13
Flounder (*Paralichthys dentatus*)	10	5*	0.16	0.14
Toadfish (*Opsanus tau*)	2	2	0.19	0.14
Eel (*Anguilla rostrata*)	1	1	—	0.15
Butter fish (*Poronotus tricanthus*)	4	2*	—	0.16
Sea robin (*Prionotus carolinus*)	5	4*	0.18	0.16
Menhaden (*Brevoortia tyrannus*)	19	8*	0.19	0.18
Sea bass (*Centropristus striatus*)	2	2	—	0.19
Porgy (*Stenotomus chrysops*)	8	3*	—	0.19
King mackerel (*Scombermorus cavalla*)	5	2*	0.21	0.19
Blue fish (*Pomatomus saltatrix*)	6	4*	0.25	0.20
Tautog (*Tautoga onitis*)	23	8*	0.27	0.21
Mackerel (*Scomber scombrus*)	65	10*	0.26	0.26

* Some measurements were made from pooled blood.

TABLE V

Molar values of isotonic solutions for elasmobranch red cells

Name of fish	Number studied	Isotonicity Molar NaCl	Elasmobranch soln.	Knowlton's soln.	Van't Hoff soln.	Lutz soln.
Smooth dogfish (*Mustelis canis*)	4	0.35	Hyper	Hypo	Hyper	Hypo
Sting ray (*Dasyatis centrura*)	2	0.33	Hyper	Hypo	Hyper	—
Skate (*Raja erinacea*)	1	0.34	Hyper	Hypo	Hyper	—
Sand shark (*Carcharias littoralis*)	1	0.35	—	—	—	—
			0.46*	0.41*	0.43*	

* Molar value of NaCl solutions of equivalent osmotic pressure as determined by ΔT_f measurements.

Biological Laboratory for use with elasmobranchs are evaluated. The value of the equivalence in moles of NaCl (obtained from the freezing point depression) of these solutions is also indicated. These data show that the range found in this group of fishes is from 0.33 *M* for the sting ray to 0.35 *M* NaCl for the sand shark and the dogfish. The range is very much less than that of the teleosts. The elasmobranch and Knowlton's solutions contain urea which explains the very large discrepancy between the evaluation of the isotonicity and the ΔT_f as discussed above.

CONCLUSIONS

The results require a closer appraisal of the physiological conditions for fish tissues suspended in artificial environments. This is particularly so for the teleosts since their bloods exhibit highly variable osmotic pressures. If the generally assumed value of 0.25 *M* NaCl were used, isotonicity would be approached only in the case of the mackerel. For the other teleosts examined, 0.25 *M* NaCl would be hypertonic and in some cases extremely so (as much as 250 per cent above normal for the shark sucker).

Although the elasmobranchs do not display the range in variation of isotonic values as do teleosts, it is important to realize that only two-thirds of the osmotic pressure of the blood is due to salts, urea being in large measure responsible for the remaining third. Thus, if 0.5 *M* NaCl were employed as a suspension medium, elasmobranch cells would be at a volume some 20 per cent lower than normal.

Emphasis should again be placed upon the very broad range of isotonic values found within the teleosts. This means that some teleosts have an osmotic pressure of their blood below that of some Amphibia. The indication is that the teleosts, considered as a group, have a more variable regulatory system than the higher groups of vertebrates as regards the solute concentration of the blood.

SUMMARY

1. Isotonic values of the blood of two groups of marine fishes were determined by three physiological methods. These were found to yield approximately the same results.

2. The freezing point depression was used to calculate isosmotic values of NaCl which proved to be generally hypertonic for the associated bloods.

3. The variation of isotonicity in the 14 teleosts examined ranged from 0.26 *M* NaCl for the mackerel to 0.10 *M* NaCl for the shark sucker.

4. Four different species of elasmobranchs were found to have isotonic values of the blood between the limits of 0.33 and 0.35 *M* NaCl. In addition, other commonly used artificial media were evaluated with respect to red cell isotonicity.

5. The extreme variability in the salt concentration of teleost blood, as determined by isotonic studies, emphasizes the need for further study of water regulation in this group of animals.

LITERATURE CITED

BAGLIONI, S., 1906. Beiträge zur allgemeinen Physiologie des Herzens. Der Einfluss der chemischen Lehensbedingungen auf die Tätigkeit des Selachierberzens. *Zeitschr. f. Allgem. Physiol.*, **6**: 71–98.

BALDWIN, E., 1949. An introduction to comparative biochemistry. Third Edition. Cambridge, England. The University Press.

BOTAZZI, F., 1897. La pression osmotique du sang des animaux marins. *Arch. Ital. de Biologia.*, **28**: 61–72.

DAVSON, H., 1951. A textbook of general physiology. The Blakiston Co., Philadelphia.

FINDLAY, A., 1941. Practical physical chemistry. Seventh Edition. Longmans, Green and Co., New York.

FREDERICQ, L., 1901. Sur la concentration moleculaire du sang et des tissus chez les animaux aquatiques. *Bull. Acad. Sci. Belgique.*, pp. 428–434.

FRY, H., 1909. Blood platelets and the coagulation of the blood in marine chordates. *Folia Hemat.*, **8**: 467–501.

GARREY, W. E., 1905. The osmotic pressure of sea water and of the blood of marine animals. *Biol. Bull.*, **8**: 257–270.

GREEN, J. W., AND J. F. HOFFMAN, 1951. Erythrocyte volume and urea in elasmobranchs. *J. Cell. Comp. Physiol.*, **37**: 506–508.

HEILBRUNN, L. V., 1952. An outline of general physiology. Third Edition. W. B. Saunders Co., Philadelphia.

KROGH, A., 1939. Osmotic regulation in aquatic animals. Cambridge, England. The University Press.

LUCKE, A., AND M. MCCUTCHEON, 1932. The living cell as an osmotic system and its permeability to water. *Physiol. Rev.*, **12**: 68–139.

LUTZ, B. R., 1930. The effect of adrenalin on the auricle of elasmobranch fishes. *Amer. J. Physiol.*, **94**: 135–139.

Manual of Formulae and Methods III, 1936. Marine Biological Laboratory Chemical Room. Woods Hole, Mass.

PARPART, A. K., 1931. Is osmotic hemolysis an all or none phenomenon? *Biol. Bull.*, **61**: 500–517.

PARPART, A. K., 1935. The permeability of the mammalian erythrocyte to deuterium oxide (heavy water). *J. Cell. Comp. Physiol.*, **7**: 153–162.

PARPART, A. K., AND R. BALLENTINE, 1943. Hematocrit determinations of relative cell volume. *Science*, **98**: 545.

PARPART, A. K., AND J. W. GREEN, 1951. Potassium and sodium exchanges in rabbit red cells treated with n-butyl alcohol. *J. Cell. Comp. Physiol.*, **38**: 347–360.

PROSSER, C. L., *et al.*, 1950. Comparative animal physiology. W. B. Saunders Co., Philadelphia.

RODIER, M. E., 1899. Observations et experiences comparative sur l'eau de mer, le sang et les liquides internes des animaux marines. *Bull. Station Biol. d'Arcachon*, **4**: 103–123.

SCHLIEPER, C., 1930. Die Osmoregulation wasserlebender Tiere. *Biol. Rev.*, **5**: 309–356.

SCHRAEDER, W., 1890. Über die Harnstoffbildung der Haifische. *Zeitschr. f. Physiol. Chem.*, **14**: 576–598.

SMITH, H. W., 1929. The composition of the body fluids of the elasmobranchs. *J. Biol. Chem.*, **81**: 407–419.

SMITH, H. W., 1932. Water regulation and its evolution in fishes. *Quart. Rev. Biol.*, **7**: 1–26.

WILLIAMS, M. M., AND M. H. JACOBS, 1931. On certain physiological differences between different preparations of so called "chemically pure" sodium chloride. *Biol. Bull.*, **61**: 485–496.

THE FATE OF RADIOPHOSPHORUS INGESTED BY HABROBRACON FEMALES[1, 2]

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In a previous publication the authors introduced the subject of the effects of phosphorus-32 on the development and heredity of offspring from adult insects given one feeding of the radioactive material (Grosch and Sullivan, 1952). The organism considered was the "ectoparasitic" wasp known in genetic literature as *Habrobracon juglandis* (Ashmead). Attention was directed to the curves representing daily egg production and to the hatchability of the eggs laid by samples of wasps after feedings at various levels of radioactivity. Subsequently we have attempted to clarify dosage relationships. In particular our desire has been to arrive at a basis for determining the dosage of radiation to which an egg may be subjected, and the present publication is concerned with some of the complications which need to be considered.

Two sources of beta radiation contribute to the dosage to which the egg contents are exposed: (1) the P^{32} in the soma of the female, and (2) the P^{32} herein shown to become incorporated into the egg. Therefore, in reference to (1), the present investigation of the consequences of ingestion of radiophosphorus was concerned with (a) observing change in the detectable radiation from wasps per unit time, an index of which is the biological half-life, and (b) illustrating changes in the distribution of radiophosphorus over the significant part of the ovulation period. Demonstrated in reference to (2) are (a) the channels whereby P^{32} may be lost by a female wasp and (b) the period during which a relatively high egg radioactivity obtains. Accordingly we are presenting the first study on insects which gives detailed chronological attention to excretory and ovipositional loss of radioactive substance subsequent to a single feeding of such material.

In addition to contributing toward a general understanding of what happens to ingested radioactive phosphorus, the present paper provides an explicit explanation of the upward slope of hatchability curves in the first week shown in the preceding report (Grosch and Sullivan, 1952). In reference to the 1952 report it should be mentioned that millicuries were erroneously indicated in the legends of several figures. Microcuries were intended throughout.

Materials and Methods

Virgin females from Habrobracon Stock No. 33 were fed mixtures of honey and P^{32} at various levels of radioactivity lower than the sterilization dose of 200 μc

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[2] Contribution from the North Carolina Agricultural Experiment Station, Raleigh, N. C. Published with the approval of the Director of Research as Paper No. 485 of the Journal Series.

per gram of mixture. Each female was given one feeding only. Biological aspects of ingestion experiments and feeding technique have been treated in detail previously (Grosch and Sullivan, 1952).

After feeding, information on the distribution and excretion of radioactive phosphorus at desired intervals was obtained from the counting equipment of the Department of Experimental Radiology, Marine Biological Laboratory, Woods Hole, Massachusetts. In particular we wish to thank Mones Berman for helpful advice and direction. Counts were determined thrice in reproducible position for each specimen. Thus the starting point of analysis was a calculation of the average of three determinations.

Nearly 300 wasps were employed in obtaining daily measurements of radioactivity on the following: (1) whole animals, anaesthetized or dead, (2) separated parts, anterior and abdomen after transection at the petiole, (3) ovaries, a few dissections only, (4) eggs, in groups and as individuals, and (5) excreta. In following daily change in the radioactivity of the organism or in checking the radioactivity of a part or product, the wasps were handled in groups of various sizes as will be detailed below when presenting results.

The technique devised for investigating the radioactivity of excreta is new. It involves maintaining the female in a punctured No. 3 gelatin capsule with one host caterpillar (*Ephestia*). The puncture in the capsule wall seemed necessary to avoid oxygen depletion. Periodic transfer of the female is made at desired time intervals, one day in the present study, whereupon female and caterpillar with deposited eggs are removed from the capsule. The caterpillar is examined microscopically for purposes of transferring any excreta (by dissecting needle) from caterpillar to the capsule thus adding to the excreta deposited on the walls. The capsule is then digested in dilute HCl in a steel planchette. After dissolution the sample is evaporated and counted.

Radioautographs made in our laboratories by Mrs. Margaret T. MacLeod have proved helpful in interpreting distribution results and reference is made to them below. Standard procedures recommended by the Eastman Kodak Company were employed in making the autographs on their Nuclear Track Plates (beta) of the 1×3 in. size with $10\,\mu$ emulsion thickness.

Results

Radioactivity of the organism

The time taken for the radioactivity of a wasp to fall to half of its initial value as the result of elimination and radioactive decay has been determined to be between four and five days as shown in Figures 1 and 2. This convenient index is known as the biological half-life. In addition, other details pertaining to change in radioactivity of a wasp can be discussed in reference to the figures.

Figure 1 presents comparatively the loss in radioactivity for three categories of whole animals: (1) killed immediately, (2) living but not ovipositing, and (3) living and ovipositing. Wasps of category (2) are infrequently seen and their ascribed lines are based only upon single animals. Also as shown by the ending of the pertinent lines, the life-span of non-laying wasps is short. The curves for non-layers are very useful in demonstrating the relatively slow loss of radioactivity when eggs are not being deposited. In categories (1) and (3) results for six

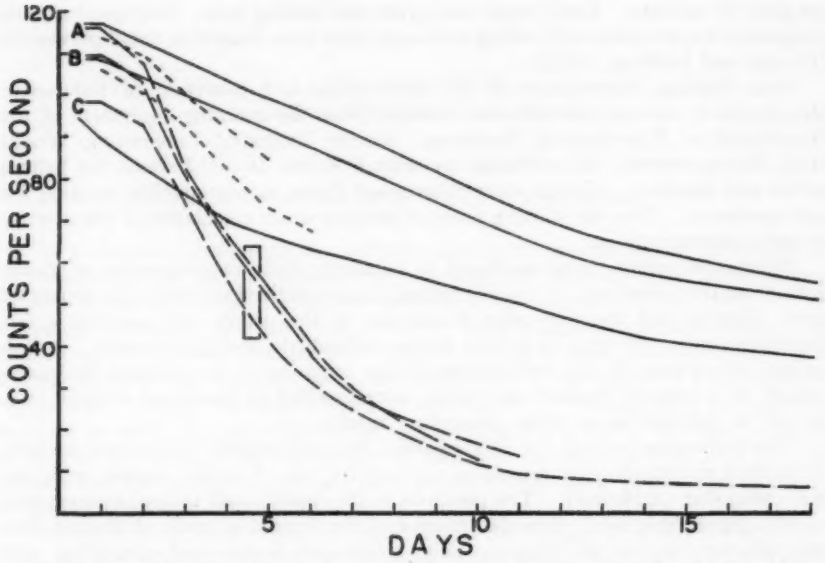

FIGURE 1. The radioactivity in mean counts per second when adult female Habrobracon are followed subsequent to ingestion of P^{32}. The solid lines are for wasps killed immediately after the single feeding. The short dashes are for live wasps not depositing eggs. The long dashes are for live wasps which are depositing eggs. The designation of the curves as A, B, and C on the basis of initial count is explained in the text. The rectangle drawn between the fourth and fifth day marks the time at which biological half life has been attained.

killed and nine ovipositing wasps, respectively, were obtained. The data from individuals in each category were grouped separately according to the level of radioactivity found in their initial counts:

Group	Initial count (c/sec.)	Individuals per group (1) Killed	(3) Ovipositing
A	110–119	2	3
B	100–109	2	3
C	90– 99	2	3
		6	9

The six curves obtained by plotting the group means on successive days are shown in Figure 1. Since all females were allowed to feed once from the same honey-P^{32} mixture, the different levels of radioactivity found in initial counts are ascribed to chance variation.

To obtain merely a decay-plus-loss curve for ovipositing animals, all nine ovipositing animals could have been lumped and the mean value compared with that from a grouping of the six dead wasps. However such a procedure obscures the detail of the crossing of lines A and B shown between days 7 and 8. An explanation of this detail seems to lie in the number of eggs deposited. Whereas the wasps represented by line A had deposited an average cumulative total of 63

eggs per animal by the sixth day, those of line B had deposited only 61.6 eggs per animal. Line C which runs almost parallel to B represents females of quite similar egg productivity (59.9 eggs, cumulative total per animal). The implication of course is that the more eggs an animal lays per unit time, the more rapid may be its loss of radioactivity. The difference in slope between non-laying and laying live wasps points to the egg as a highly significant channel of P^{32} loss. Direct evidence of egg radioactivity is treated in the following section.

Figure 2 presents results based on larger numbers of animals in verification of the biological half-life. Thirty live ovipositing animals were followed for seven days. These animals comprised three groups which reflect the feeding of three

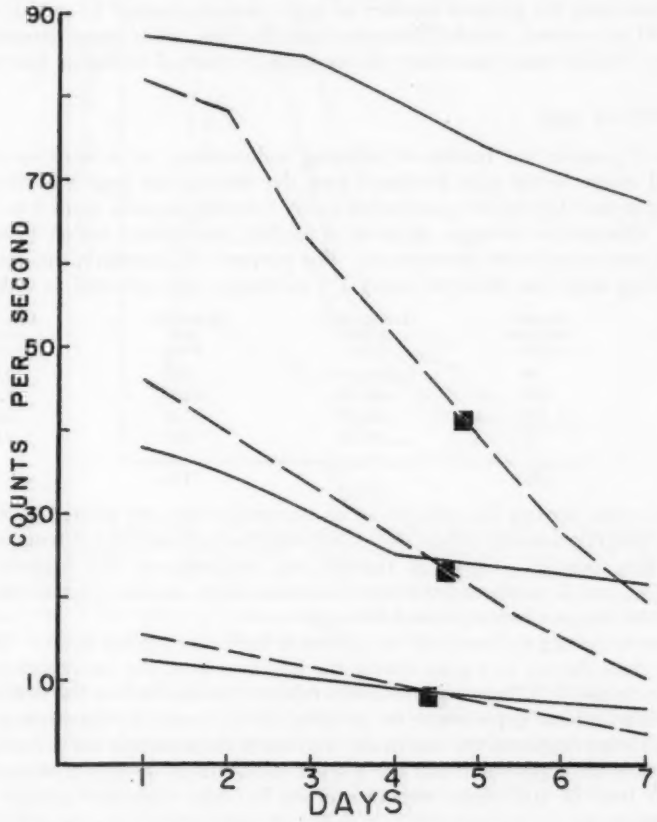

FIGURE 2. Verification of the results of Figure 1 using larger samples of wasps at three levels of radioactivity. The solid lines are for wasps killed immediately after feeding. They demonstrate measurable decay of P^{32} with a half-life extrapolating to between 14 and 15 days. The broken lines refer to egg-laying wasps with squares drawn to mark biological half life reached between four and five days.

different radioactive honey mixtures. Ten wasps were obtained therefrom with initial counts of less than 30 per second, 10 between 30 and 60 counts per second, and 10 between 60 and 100 counts per second. The group means of these live ovipositing wasps have been plotted as three broken lines to be compared with the solid lines which represent the mean values for 7 killed wasps numbering 2, 2 and 3 dead individuals per respective range of initial count. Here too it is demonstrated that the total radioactivity of an animal is related to the number of eggs deposited. By the fourth day the wasps counting less than 30 c/second had deposited an average total of 33.4 eggs per animal, those between 30 and 60 c/second had deposited an average total of 31.5 eggs per animal and the 60 to 100 c/second group had deposited an average total of only 24.2 eggs per animal. As indicated in Figure 2 the animals depositing the greatest number of eggs (wasps grouped by initial count of less than 30 per second) reached biological half-life first, while those depositing the fewest eggs (initial count more than 60 per second) reached biological half-life last.

Radioactivity of eggs

Figure 3 presents the results of following radioactivity for a week or more by taking GM counts of the eggs produced each day during that length of time. Depending upon the stage of her production cycle, a female deposits from 5 to 20 eggs per day. This number of eggs, whatever it totalled, was spread out on a planchette and counts per second were determined. For purposes of summary, the data which reflect feeding with four different honey-P^{32} mixtures, were grouped as follows:

Group	Number wasps per group	Initial count of wasps c/sec.	Total eggs per group	Max. level c/sec. per egg
A	9	above 90	393	10.99
B	19	60–90	1403	7.28
C	17	30–59	1133	5.32
D	5	5–15	819	2.09
Totals	50		3748	

The mean counts per egg for each group on successive days are plotted in Figure 3. A very definite relationship is thus shown between the radioactivity of females and of the eggs they deposit. Curve A reaches and continues at the highest values. Curves B, C, and D reach and continue at lower values in the order of the radioactivity of the wasps which deposited the eggs.

The curves for egg radioactivity are shown to have very typical shape. The level of activity rises sharply to a peak during the first two days, the maximum level obtained in each case. It then drops toward a relative low reached on the fifth or sixth day. In an additional experiment, not graphed, daily counts on eggs from a sample of 21 wasps were begun on the fourth day and made subsequently for a week, which proved tenable the supposition that the level of radioactivity of eggs continues to fall slowly. A total of 1075 eggs was considered by daily deposited groups in this experiment.

In further experiments, counts were made on single eggs of all those deposited by individual wasps on particular days subsequent to the ingestion of radioactive material. This was an attempt to obtain information on the variability in radioactivity of eggs within given days. Records of the radioactivity of 212 eggs were ob-

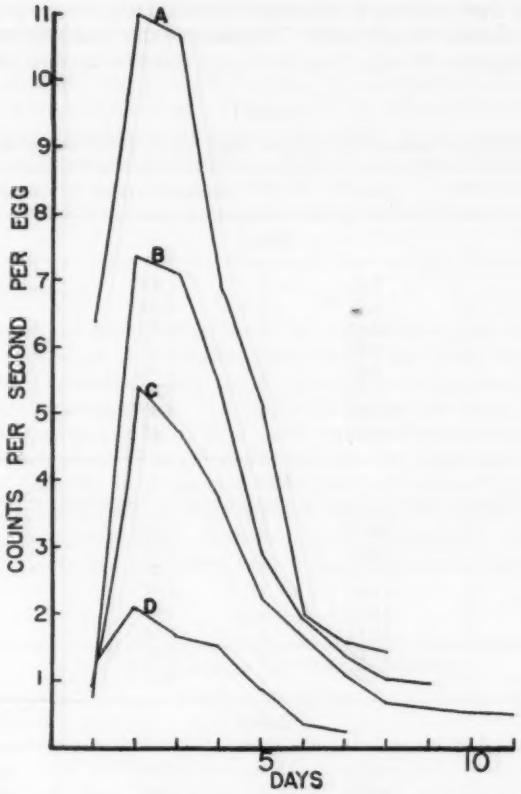

FIGURE 3. The radioactivity of Habrobracon eggs laid by samples of wasps after feeding once on honey containing P^{32} at zero days. Each line represents calculations of the arithmetic average for single eggs derived from GM counts per second on accumulations of eggs representing the daily production from individual wasps. The different level reached on day two reflects the initial level of radioactivity of the wasps depositing the eggs, and the designation A, B, C, and D is in order from high to low.

tained, representing conditions over the first five days of egg production. A variety of dosages were considered which resulted in wide differences in the means. The mean itself was not of primary interest here but rather the degree of variation around the mean to be represented as a comparative or relative measure. Such a measure is furnished by the coefficient of variation. Summarization is given in Table I. It will be noted that the comparisons are made between samples of approximately the same size, hence it is unlikely that S.D.'s and C.V.'s are varying because of chance inclusion of extreme variates in unequal sized samples. On the first day there is appreciable variation in radioactivity as shown by standard errors which are relatively large when compared with the mean. This is expressed in consistently higher coeffi-

cients of variation than are seen in subsequent days. Data for second and third days show variability similar to each other. Because of the time-consuming aspects of this type of investigation the eggs from few wasps could be handled on the fourth and

TABLE I

Summary of radioactivity data for single eggs in counts per second

Number of eggs	Mean	Standard deviation	Coefficient of variation
	Day 1		
5	3.97	1.57	39.5
7	8.41	3.06	36.3
7	2.68	0.92	34.3
5	6.49	2.17	33.4*
7	8.45	2.36	27.9
7	2.78	0.58	20.8
5	37.96	5.80	15.3
5	24.46	2.47	10.1
	Day 2		
9	14.18	3.09	21.8*
6	10.13	1.33	13.1
6	10.13	1.16	11.5
8	11.42	1.07	9.3
8	11.41	1.03	9.0
11	12.57	1.04	8.2
12	12.46	1.01	8.1
5	14.40	0.75	5.2
	Day 3		
9	11.39	2.99	26.2*
11	10.79	2.62	24.2
8	45.61	5.96	13.0
14	20.10	2.36	11.7
8	13.18	1.22	9.2
4	36.85	3.02	8.2
8	30.62	1.89	6.1
	Day 4		
6	9.27	2.15	23.1
4	46.05	5.19	11.2
5	5.96	0.62	10.4*
3	10.91	0.66	6.0
8	6.33	0.26	4.1
	Day 5		
6	5.69	0.62	10.9
6	6.53	0.27	4.1*

fifth day; too few to permit conclusions to be drawn. After the fifth day, individual eggs count so very slowly that investigation of the radioactivity of single eggs had to be discontinued.

Admittedly it would have been ideal to have all records provided by the same animals followed for the whole period. However, in the absence of an automatic feeder device for the equipment used to measure radioactivity, it was humanly impossible to carry out the ideal design of experiment. Only in one case were the eggs from the same animal examined over the whole five day period. These are indicated on the Table by asterisks. Here there is suggested progressively less variation in successive days.

Radioactivity of excreta

The radioactivity of the excreta collected by the gelatin capsule method was measured daily for each wasp of a sample of twenty-five fed from the same honey-P^{32} mixture. Before and after the four-day duration of the experiment the radioactivity of each wasp was also measured. By correcting for isotope decay, the initial radioactivity of each wasp and of its excreta were put on a common basis with the final radioactivity of each wasp. The amount of loss was then calculated (by subtraction) for comparison with the total amount (addition) of radioactivity of the excreta. Table II presents a summary of these data for actively ovipositing wasps and gives the comparison as a percentage. Since it was the comparison which was particularly desired the data are organized with reference to percentage by a listing in

TABLE II

A comparison of the radioactivity of excreta with the total radioactivity lost in four days by ovipositing Habrobracon

Total radioactivity of excreta in c/sec. corrected for decay	Loss in radioactivity not due to decay c/sec.	Per cent of loss through excreta
10.18	48.22	21.1
5.81	25.11	23.1
10.78	44.63	24.2
9.47	36.95	25.6
15.67	55.76	27.6
19.69	62.75	31.4
9.59	29.51	32.5
5.94	17.30	34.4
11.99	30.55	39.2
10.04	25.47	39.4
7.99	19.93	40.1
10.47	25.98	40.3
15.41	37.81	40.7
23.25	55.96	41.5
22.00	52.70	41.7
8.03	17.61	45.5
9.42	19.69	47.8
5.67	11.51	49.2
7.36	14.57	50.5
13.02	24.20	53.7
19.93	36.82	54.2
25.27	43.49	58.1
8.38	13.75	60.9

order from the lowest to the highest. A consideration of the distribution of percentages in Table II demonstrates that the mean, median and mode fall near 40%, a value which may be taken provisionally to represent the average loss of radioactive phosphorus to be expected to occur by way of the excreta for ovipositing females. Data for two females which laid few or no eggs have been omitted from the Table. Excreta accounted for 82% and 100% of their radioactive loss.

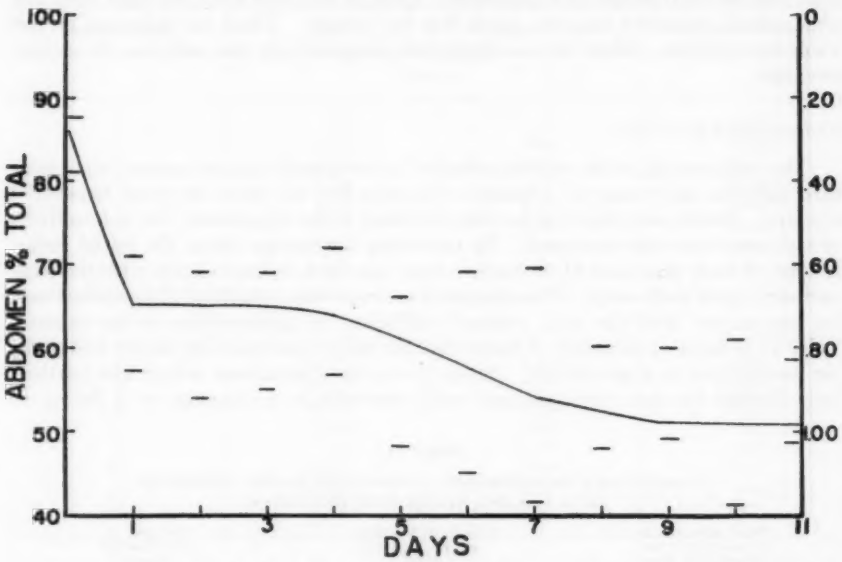

FIGURE 4. The distribution of P^{32} in Habrobracon females subsequent to a single feeding of the honey mixture. The mean value is plotted for the per cent of the total radioactivity demonstrable for the abdomen and the daily range is deliminated by dashes. Included on the right is a scale for interpreting the same results in terms of an anterior/posterior ratio.

Distribution of P^{32}

Transection experiments summarized in Figure 4 demonstrate that shortly after feeding, the abdomen contains a major amount (86%) of the radioactive material. This of course is to be expected since the crop, an organ which functions in immediate storage of food, has an abdominal location. Within twenty-four hours the proportionate distribution has changed so that only about 65% of the active material is abdominal. As shown, this situation remains constant during four successive days corresponding to the period during which the biological half-life is reached and during which appreciable amounts of radioactive material are egested and incorporated into deposited eggs. Subsequently, the abdominal radioactivity falls off so that from the seventh day forward there is only slightly more than 50% of the total radioactivity demonstrable as abdominal in a majority of animals. These conclusions are based upon transections of 134 females, of which 5 to 15 were sacrificed per day. Eggs laid each day were recorded and cumulative

egg production for each female was calculated therefrom. Although oviposition has been shown above to be a very significant channel for exit of radioactive material and therefore a very important factor in the regulation of the total radioactivity of the animal, egg laying does not appear to be significant in determining P^{32} distribution.

Attempts to show a correlation between total eggs produced and proportionate distribution of P^{32} have failed. The evidence as given by scatter diagrams will not be presented. Instead the following selected examples will serve to demonstrate the point that although there may be appreciable differences in the number of eggs laid by females, however, provided an equivalent amount of time has elapsed since feeding, a very similar distribution of radioactive material may be expected:

Day	Abdominal per cent of radioactivity	Cumulative total eggs
7	53.67	94
	53.62	115
9	52.73	98
	52.82	56
11	52.23	183
	52.40	71

Apparently egg production is much less important in bringing about distribution than uninvestigated non-specified metabolic activities which appear to require elapsed time. Consistent with these conclusions is the fact that ovaries dissected out of adult females show very slow counts after biological half-life has been passed. In the present investigation, dissected ovaries were found to contain less than 33% of the abdominal radioactivity and one egg-laying female on the eighth day carried only 7% of her abdominal radioactivity in the ovaries.

The conclusion that physiological activities other than egg production are importantly involved in the distribution of radioactive phosphorus raises the question of where the material is present. The gonads apparently do not function as a storage depot but rather as disposal units which draw upon the reservoirs in the organism for the P^{32} to be incorporated into eggs. Thus distribution of radiophosphorus in Habrobracon is viewed as mobile and adjustable, an approach entirely acceptable on the basis of turnover studies in other organisms. One of the most definite of the general results emerging from tracer research is the demonstration of the dynamic character of molecules involved in metabolism (Kamen, 1947).

Radioautographs of Habrobracon females fed P^{32} are in accordance with these views in showing the trophocytes as the most radioactive elements of the gonads, while in addition other wasp tissues become particularly radioactive such as the muscles, the midgut epithelium, the brain and the central nervous system. Observations of radiophosphorus content of the tissues enumerated are consistent with reports on radioautographs of other insects (Lindsey and Craig, 1942; Irwin, Spinks and Arnason, 1950). Incidentally, because we are using P^{32} in amounts larger than tracer doses, the density of silver granules is great and resolution is lacking for some time after feeding. In fact, distinguishable details on wasp radioautographs were not clear unless the animal was sacrificed several days after feeding and our best radioautographs were made after biological half-life. The only detail discernibly distinct in radioautographs made one day after P^{32} feeding is

the lack of radioactivity for the contents of the digestive tract in the region of the crop and stomach.

Discussion

A consistent picture emerges here of the loss of most of an ingested quantity of P^{32} within a few days after a single feeding of the radioactive material. Furthermore three lines of evidence point to the egg as a major channel whereby the isotope is eliminated from the fed female. In the first place, a comparison of curves in Figure 1 indicates the rapidity with which ovipositing wasps lose radioactivity in comparison to non-laying wasps. Secondly, the measurements of excretal radioactivity demonstrate that excretory deposits account for only around 40% of the loss in radioactivity. Finally, there is direct evidence from the demonstrated radioactivity of the eggs themselves. Thus an individual egg from a wasp fed P^{32} is subjected not only to the beta rays from the radioactive tissues and body fluids before laying, but irradiation is continued after deposit from the P^{32} incorporated into the egg.

Eggs laid the first day may be expected to have been in Metaphase I, a susceptible stage (Whiting, 1945), and hence influenced by maximum exposure to emanations from the soma of the mother. However, those laid subsequently up to about the sixth day were progressing through Prophase I, a relatively resistant stage, when the mother is most radioactive (Day 1). A great difference has been demonstrated between the eggs of day 2 and those of day 5 in the amount of egg-incorporated radiophosphorus. Curve A of Figure 3 shows that eggs on the second day after feeding can have as much as seven times the incorporated amount of radioactive material demonstrable a few days later. The period when incorporated P^{32} is at a high level is also when hatchability was found to be low particularly after a feeding of highly radioactive mixture (Grosch and Sullivan, 1952). Subsequently in the period when radioactivity falls off, the hatchability increases appreciably. We therefore believe now that egg radioactivity is most important in explaining hatchability during the 1- to 5-day period.

Eggs laid after the fifth day are not appreciably radioactive. Either initial exposure as mitotically active oogonia or total accrued dosage may be more significant in influencing their hatchability than the incorporated amount of radioisotope. In any event, it has become evident that eggs laid at different times by the same radiophosphorus-fed animal become exposed to quite different dosages of beta radiation from various sources.

The present authors are especially impressed with the concept of a gamete which carries built-in radiation equipment of particular potency in eggs laid early in the first week. Any susceptible stage in oogenesis or embryological development subsequent to incorporation may be subjected to ionizing radiations from a most intimate source, perhaps even from the chromosomes themselves.

There may be important genetic implications of the present results in an extension to what may occur in economically important vertebrates. For one thing, large amounts of radioactive phosphorus have been demonstrated to be removed from the physiological interior of the domestic fowl by way of the egg (Lorenz *et al.*, 1942). However attention has apparently not been directed to either the hatchability or the mutational potentialities of such radioactive products. Instead, interest has been devoted to applying tracer techniques to the physiological aspects

of the treated adult rather than to investigating developmental or genetical consequences in the offspring (Entenman *et al.*, 1938; Hevesy, 1948; Lorenz *et al.*, 1942).

On the other hand, since there is no equivalent shell featuring inorganic constituents, it does not seem profitable to anticipate Habrobracon physiology on the basis of poultry work except in broad general terms. If the extension from vertebrate to invertebrate is valid, the element would soon make its appearance as organic phosphorus and as such be incorporated into eggs. The two days between feeding and the peak radioactivity of eggs seem to be an adequate period elapsed for synthesis.

Summary

1. The biological half-life of radiophosphorus in egg-laying Habrobracon females is four to five days.

2. A majority of the P^{32} lost, about 60%, is by way of deposited eggs. This is based upon excretory studies using a newly devised technique, and upon comparisons of laying and non-laying wasps. Direct evidence of the radioactivity of the eggs is also presented.

3. The radioactivity of eggs rises to a peak reached the second day after feeding. Subsequent to the third day, it drops sharply to a relatively low plateau.

4. The variability in radioactivity from egg to egg is greatest in the first day. It may prove to be less each succeeding day.

5. During the first day almost 90% of the ingested radioactive material is abdominal due to the location of the crop. From the second to the fourth day about 65% of the radioactivity in the female is abdominal. Subsequently abdominal radioactivity approaches 50% of the total. Of these proportions less than one-third may be gonadal.

6. Although it is an important factor in the regulation of total radioactivity, egg laying has not been found significant in determining P^{32} distribution. A mobile, adjustable situation is indicated.

7. The shape of the hatchability curve previously reported for eggs from females fed P^{32} is interpreted for the first five days on the basis of gamete-incorporated radiophosphorus. Subsequently, initial exposure and accrued total dose may have greater significance.

LITERATURE CITED

Entenman, C., S. Ruben, I. Perlman, F. W. Lorenz and I. L. Chaikoff, 1938. Radioactive phosphorus as an indicator of phospholipid metabolism. III. The conversion of phosphate to lipoid phosphorus by the tissues of the laying and non-laying bird. *J. Biol. Chem.*, **124**: 795–802.

Grosch, D. S., and R. L. Sullivan, 1952. The effect of ingested radiophosphorus on egg production and embryo survival in the wasp Habrobracon. *Biol. Bull.*, **102**: 128–140.

Hevesy, G., 1948. Radioactive indicators. Interscience Publishers, Inc., New York.

Irwin, R. L. B., J. W. T. Spinks and T. J. Arnason, 1950. Deposition of P^{32} in developing Drosophila. *Canadian J. Res., D*, **28**: 137–142.

Kamen, M. D., 1947. Radioactive tracers in biology. Academic Press, Inc., New York.

Lindsey, E., and R. Craig, 1942. The distribution of radiophosphorus in wax moth, mealworm, cockroach and firebrat. *Annals Ent. Soc. Amer.*, **35**: 50–56.

Lorenz, F. W., I. Perlman and I. L. Chaikoff, 1942. Phosphorus deposition in the egg as measured with radioactive phosphorus. *Amer. J. Physiol.*, **138**: 318–327.

Whiting, A. R., 1945. Effects of X-rays on hatchability and on chromosomes of Habrobracon eggs treated in first meiotic prophase and metaphase. *Amer. Nat.*, **79**: 193–227.

STUDIES ON THE INTEGUMENT OF THE SILKWORM, BOMBYX MORI. VII. THE PERMEABILITY OF THE INTEGUMENT TO OXYGEN AND CARBON DIOXIDE IN VIVO

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The fact that arthropod cuticle is permeable to gases, such as O_2 or CO_2, has been known in various materials (von Buddenbrock and von Rohr, 1922; Maloeuf, 1936; Fraenkel and Herford, 1938; and see the review by Richards, 1951). But in the silkworm any research on this respect is not found. The experiments reported here were undertaken to get quantitative data on the permeability to O_2 and CO_2 in the silkworm and, further, to know whether or not there is some difference in this property between the larva and the pupa.

On the other hand, that the insect cuticle, especially the epicuticle, is waterproof is a well known fact in general (Wigglesworth, 1948; Richards, 1951) and a procedure, abrasion of the cuticle with alumina dust, has been employed in the study of the transpiration of water through the cuticle. In the present research the effect of the same treatment on the permeability to O_2 and CO_2 was examined. Further, some other effects of rubbing are described here preliminarily.

Materials and Methods

Two silkworm races, C122 × N122 and Taihei × Chôan, reared in the spring of 1952, were used as material. Oxygen uptake and CO_2 output were measured manometrically by Warburg apparatus with the materials operated as follows: (1) Normal larvae and pupae. The larvae were ligated immediately behind the head in order to avoid extrusion of the digestive fluid from the mouth, and at the level between the eighth and the ninth abdominal segment in order to prevent the excretion of excrements. (2) Sealed larvae and pupae. In the former all spiracles, and the parts of the body both anterior to the head ligation and posterior to the abdominal ligation were sealed with enamel paint, and in the latter all spiracles, the segments behind the seventh abdominal segment and the skin at the boundary between wings and body were sealed with it. Materials were placed in the vessels of the manometer 15 minutes after occlusion in the larva and 20 minutes after occlusion in the pupa. Readings were begun after a 15-minute equilibration period. Controls showed that the paint had lost its volatile components before measurements were made, and that neither the paint nor the ligation significantly affected the results. To measure CO_2 output, the method comparing O_2 uptake in the presence or absence of alkali was used. Besides 0.3 ml. of 10% KOH placed in the center well, 0.5 ml. of about 10% H_2SO_4 was placed in the side arm of the vessel for the purpose of taking up possible ammonia there. All experiments were carried out at 25° C. and the gas phase was air.

For certain experiments the surface of the cuticle was abraded by scattering alumina powder on hard paper and then rubbing the worm lightly against this repeatedly.

The time course of O_2 uptake did not show a linear relation but a slightly upward convex curve in the sealed worms, showing that the rate of O_2 uptake gradually decreased, as shown in Figure 1. This was also the case in CO_2 output. As it seemed possible to get more reliable and mutually comparable values in O_2 uptake and CO_2 output at the beginning of the measurement, μl. O_2 uptake or CO_2 output per gram live weight in the first 30 minutes was used as a measure of the amount of gas penetration through the skin. The number of sealed silkworms used varied from three to five for one vessel, and two to four measurements were repeated.

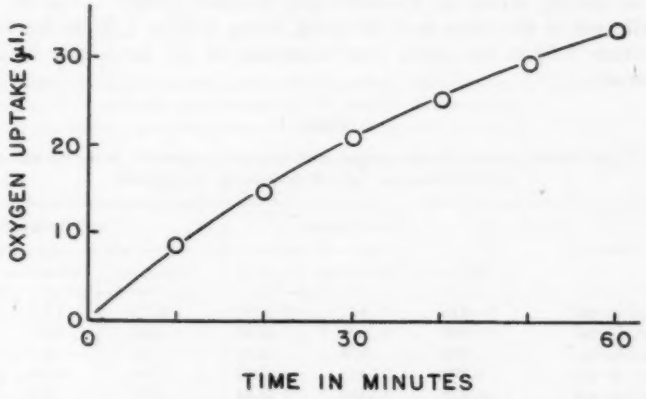

FIGURE 1. Time course of oxygen uptake of the closed silkworm larva. (μl./g. live weight).

It must be noted before the explanation of experimental results that there is no sure method of deciding whether the gasometric data obtained in the occlusion experiments are direct results of the gas penetration through the integument or not. But judging from the facts that the spiracles were perfectly occluded, that the film of the paint was considerably thick, and that there seems to be no part from which gases can penetrate out, it appears that the measured data in the occlusion experiments show practically the volume of gases penetrating through the integument.

OXYGEN UPTAKE AND CARBON DIOXIDE OUTPUT IN THE SEALED SILKWORM

The results of the experiments with fifth instar larvae as well as the pupae are given in Table I. From the values obtained in the sealed silkworms, it is obvious that the integument is permeable to O_2 and CO_2 both in the larva and pupa *in vivo*, even though the amount of gases is very much reduced in the sealed ones, to about one-fiftieth of the normals. Especially, after the middle time of the pupal stage, O_2 can be hardly measured. It has been already shown in the silkworm that the rate of the respiration varies during development (Itaya, 1940). This is shown also to be valid for the normal silkworms in Table I, where the respiratory rates similar to those obtained by him are represented, but not for the sealed ones. In other words, the rate of O_2 uptake or CO_2 output through the integument is per-

haps independent of the normal respiration. The values obtained in the sealed worms are relatively higher on the day of the larval and the pupal ecdysis, only, as compared with any other time. This may be caused by the fact that the cuticle is the thinnest at that time, both in the larva and pupa (Kuwana, 1933; Ito, 1951). However, such a phenomenon as the amount of gases passing through the integument being somewhat related to the thickness of the cuticle is not seen, after the first 24 hours of each ecdysis.

As shown in Table I, the values of the R. Q. are below 1.00 in the normal worms but considerably higher in the sealed ones (see also Table II), like those obtained in blowfly larvae by Fraenkel and Herford (1938). The R. Q. is distinctly different in the larva and the pupa, being 1.00 to 1.20 in the former and approximately 1.50 in the latter (one exception in the larva may be an experimental error).

TABLE I

Oxygen uptake, carbon dioxide output, and respiratory quotient in the normal and sealed silkworms (μl./30 minutes/g. live weight)

Stage	Normal silkworm			Sealed silkworm		
	O_2 uptake	CO_2 output	R. Q.	O_2 uptake	CO_2 output	R. Q.
1-day larva*	456	411	0.90	12.5	14.3	1.14
2-day larva	699	631	0.90	8.5	10.0	1.18
6-day larva	696	678	0.97	7.6	8.4	1.10
7-day larva	392	323	0.82	7.8	10.5	1.35
8-day larva†	428	360	0.84	9.2	9.2	1.00
10-day larva‡	241	205	0.85	9.4	10.0	1.06
1-day pupa§	190	168	0.88	12.2	19.1	1.57
4-day pupa	84	75	0.89	5.7	8.4	1.47
8-day pupa	179	165	0.92	±	11.2	—

* All larvae are in the 5th instar.
† The day of ripening (worms begin to spin cocoon).
‡ The third day after ripening.
§ Several hours after pupation.

Since the curve of the time course of O_2 uptake or CO_2 output in the sealed material is slightly convex, as shown above (Fig. 1), it is imagined that the rate of gas passage through the integument and the R. Q. value change according to the length of time after occlusion. The results of experiments in this connection are represented in Table II, in which the degree of change expressed by the ratio is also shown. In all cases the rate of O_2 uptake as well as of CO_2 output decreases and the ratio of decrease is more remarkable in the larva than in the pupa (see Fig. 2). The rate of O_2 uptake decreases less than that of CO_2 output and as a consequence the R. Q. becomes low both in the larva and pupa.[1] But several

[1] In the first-day pupa, however, somewhat increased R. Q. is gained. This seems to be caused by the presence of difficulty in manometer experiments in the time when the respiratory rate is very much depressed, in some time, to the extent of a scarcely recognizable value on the manometer.

hours after pupation (when the quinone-tanning process is not yet finished) the decrease is found relatively remarkable and is similar to that of the larva.

The rate of decrease shown in Table II is illustrated in Figure 2, in which all the data on the larval or pupal stage are collectively arranged according to the length of time after occlusion, as the degree of change in O_2 uptake or CO_2 output remains practically constant throughout larval or pupal stage. It is shown from this that the ratio decreases almost constantly according to the time after occlusion, regardless of stage. This tells us also that the amount of O_2 and CO_2 passing through the integument per gram live weight is nearly constant in the larva and pupa, respectively, except for the short period after the ecdysis, as mentioned above.

TABLE II

The change of oxygen uptake, carbon dioxide output, and respiratory quotient after occlusion of spiracles (μl./30 minutes/g. live weight)

Stage	Time in hours after occlusion*	Measured value			Ratio to the initial value (%)	
		O_2 uptake	CO_2 output	R. Q.	O_2 uptake	CO_2 output
2-day larva	0	8.5	10.0	1.18	100	100
	2	6.6	6.4	0.97	78	64
3-day larva	0	9.5	9.7	1.02	100	100
	3	4.2	3.6	0.86	44	37
5-day larva	0	11.8	12.3	1.04	100	100
	3	6.7	6.4	0.96	57	52
6-day larva	0	7.6	8.1	1.07	100	100
	1.5	6.4	5.9	0.92	84	73
1-day pupa	0	12.2	19.1	1.57	100	100
	2	8.4	14.8	1.76	59	77
4-day pupa	0	5.7	8.4	1.47	100	100
	2.5	5.2	6.7	1.29	91	80
8-day pupa	0	±	11.2	—	—	100
	3	—	9.0	—	—	80

* The second measurement of each stage was performed using the same worms.

MEASUREMENT IN THE RUBBED SILKWORM

The fact that the rate of transpiration of water is much increased when the most superficial layer of the insect cuticle is abraded by rubbing lightly with alumina dust is well established, but no information is available about the effect of rubbing on the permeability to gases. To know the effect of it in the silkworm, measurements were performed in the same way as in the preceding section, employing the worms rubbed with the powder immediately before spiracular sealing. The data are shown in Table III. From this it is obvious that when worms are rubbed, the rates of O_2 uptake and CO_2 output are increased as compared with

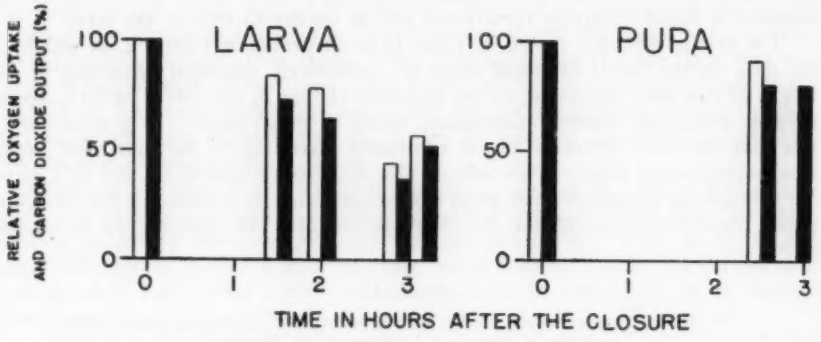

FIGURE 2. The decrease of oxygen uptake and carbon dioxide output of the sealed silkworm with the time lapse after closure (measurements for every 30 minutes). White column, oxygen uptake. Black column, carbon dioxide output.

the unrubbed worms (compare Table III with Table II). The increase of CO_2 output is proportionately one and a half times as high as the increase of O_2 uptake (Table IV). Further, the increase for both O_2 uptake and CO_2 output is a little higher in the larva than in the pupa, without exception. The fact that the increase of CO_2 output is higher than that of O_2 uptake throughout all experiments causes the value of the R. Q. to reach to a level as high as about 1.50 in the larva and more than 2.00 in the pupa.

The rate of O_2 uptake and CO_2 output decreases according to the time lapse after occlusion (Table III and Fig. 3), like the case mentioned above (Table II and Fig. 2). But the decrease of O_2 uptake is less marked than that of CO_2 output, especially in the larva. Therefore, reduction of the R.Q. value is remarkable in the larva.

TABLE III

Oxygen uptake, carbon dioxide output, and respiratory quotient of the silkworms rubbed on the cuticle with alumina dust (μl./30 minutes/g. live weight)

Stage	Time in hours after occlusion	Measured value			Ratio to the initial value (%)	
		O_2 uptake	CO_2 output	R. Q.	O_2 uptake	CO_2 output
3-day larva	0	25.6	36.1	1.42	100	100
	3	19.4	18.9	0.97	76	52
5-day larva	0	21.5	34.2	1.59	100	100
	3	19.0	16.9	0.89	88	49
1-day pupa	0	14.6	37.9	2.60	100	100
	2	10.8	23.9	2.21	74	63
4-day pupa	0	8.1	17.9	2.21	100	100
	2.5	7.2	13.3	1.85	89	74

TABLE IV

The ratio of increase of the rate of oxygen uptake as well as carbon dioxide output by abrasion in the sealed silkworms

Stage	O_2 uptake	CO_2 output
3-day larva	2.7	3.7
5-day larva	1.8	2.8
1-day pupa	1.2	2.0
4-day pupa	1.4	2.1

SOME REMARKS ON THE RUBBED SKIN

The cuticle begins to darken at the rubbed part a few hours after rubbing and becomes black or dark brown in color about 24 hours later. The color differs a little according to the different developmental stages and is similar to the color developed in the body fluid exposed to air. The darkening occurs both in the larva and pupa but there is some difference in the time before melanosis [2] according to the developmental stages. Another interesting fact observed in the rubbed integument is that the scales are not formed in the surviving adult, if the pupae are rubbed in the middle or later stage, though most of them die. However, if the rubbing is performed shortly before the larval or pupal ecdysis, the molted larva or pupa shows quite normal appearance. Histological observations will be described in the future.

DISCUSSION

From the occlusion experiment it is obvious that the integument of the larva or pupa in the silkworm is permeable to O_2 and CO_2 *in vivo*, at least in the sealed worms. There seems to be no direct way of deciding whether the cutaneous respiration reaches the observed value also in controls, but the opinion of Fraenkel and Herford (1938) that the amount of O_2 diffusing through the skin in the normal animals would be much less than in the closed animals, may be applied in the silk-

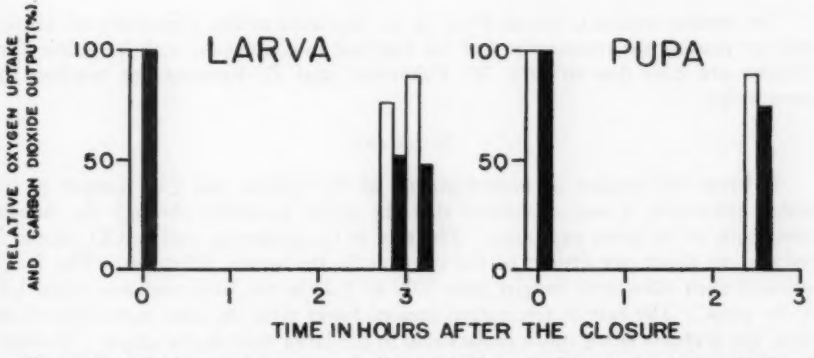

FIGURE 3. The decrease of oxygen uptake and carbon dioxide output of the rubbed and sealed silkworm with the time lapse after closure (measurements for every 30 minutes). The explanations are the same as for Figure 2.

[2] The darkening of the cuticle is considered to be a kind of melanosis.

worm also. And judging from the very low rate of O_2 uptake or CO_2 output in the sealed silkworms, that is, about one-fiftieth of the normals, it is sure that the cutaneous respiration is so slight as to be practically negligible as compared with the tracheal respiration.

In the larvae of *Calliphora erythrocephala* (Diptera), *Chearocampa elpenor* (Lepidoptera), *Tenebrio molitor* (Coleoptera) and *Culex* sp. (Diptera), the rates of respiration in the closed ones range from one-half to one-fifth of the normal animals (Fraenkel and Herford, 1938), being much higher than the values obtained in the silkworm (about one-fiftieth). This difference may be attributed to the minute structure of the cuticle, blood physiology and so forth. But O_2 uptake calculated from Table I amounts, for instance,

to 0.025 μl./mg./hr. (one-day larva of the fifth instar), or
to 0.015 μl./mg./hr. (six-day larva of the fifth instar)

in the closed worm in air, and these values do not differ, in the order, as compared with the values shown in *Calliphora* by the above two investigators. It is supposed, therefore, that the low value, such as one-fiftieth of the normal in the sealed silkworms, would be brought about because tracheal respiration is far more important in this insect than in *Calliphora erythrocephala*, etc.

In the closure experiment of *Cambarus bartoni* it is shown that the rate of respiration becomes lower with the time lapse, and that the R. Q. value becomes higher in this course (Maloeuf, 1936). In contrast to this, it is shown that the R. Q. value is reduced in the sealed silkworm, though the same change as in the former animal is seen also in the latter one as for the time course of the respiratory rate.

It was shown in other experiments that the transpiration of water is much increased by rubbing in the silkworm pupa (Ito and Tanaka, 1952). In this paper it is shown that the rate of permeability to O_2 and CO_2 is increased by rubbing, but the ratio of increase in permeability to O_2 and CO_2 by abrasion is far lower than that to water by the same procedure.

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Summary

1. From the results of measurements of O_2 uptake and CO_2 output in the sealed silkworm, it was confirmed that the gases penetrate through the integument both in the larva and pupa. The rate of O_2 uptake as well as CO_2 output is reduced to about one-fiftieth of the controls in the sealed silkworm. The R. Q. value of such silkworms ranged from 1.00 to 1.20 in the larva and was about 1.50 in the pupa. The rate of respiration became lower with the time lapse after occlusion, the decrease being more remarkable in the larva than in the pupa. Throughout the larval and pupal stages, the rate of O_2 uptake decreased less than that of CO_2 output.

2. The permeability of the skin rubbed just before occlusion increased a few times, the ratio being a little higher in the larva than in the pupa. The rate of in-

crease in CO_2 output was about one and one-half times that in O_2 uptake both in the larva and pupa. The decrease of the volume of measured gases seen with the time lapse in the rubbed material was not so remarkable in the pupa, and the rate of decrease in CO_2 output was relatively high in the larva. From this it was shown that the increase of cutaneous respiration by abrasion was not so remarkable as compared with that of water transpiration through the integument.

3. Some observations in relation to cuticle rubbing were briefly outlined.

LITERATURE CITED

VON BUDDENBROCK, W., AND G. VON ROHR, 1922. Die Atmung von *Dixippus morosus*. *Zeitschr. allg. Physiol.*, **21**: 111–160.

FRAENKEL, G., AND G. V. B. HERFORD, 1938. The respiration of insects through the skin. *J. Exp. Biol.*, **15**: 266–280.

ITAYA, K., 1940. Respiration and respiratory quotient in larva, pupa and adult of silkworm. *J. Sericul. Sci. Japan*, **11**: 113–140. (In Japanese.)

ITO, T., 1951. Studies on the integument of the silkworm, *Bombyx mori*. II. Histology and cytology of the integument at the pupation time. *Bull. Sericul. Exp. Sta.*, **13**: 585–611. (In Japanese with English summary).

ITO, T., AND M. TANAKA, 1952. Waterproofing of the pupal cuticle in the silkworm. *Sanshi-kenkyû*, **2**: 23–26. (In Japanese.)

KUWANA, Z., 1933. Notes on the growth of cuticle in the silkworm. *Proc. Imp. Acad. Tokyo*, **9**: 280–283.

MALOEUF, N. S. R., 1936. Quantitative studies on the respiration of aquatic arthropods and on the permeability of their outer integument to gases. *J. Exp. Zool.*, **74**: 323–351.

RICHARDS, A. G., 1951. The integument of arthropods. Univ. of Minnesota Press, Minneapolis.

WIGGLESWORTH, V. B., 1948. The insect cuticle. *Biol. Rev.*, **23**: 408–451.

THE ORIGIN AND DISTRIBUTION OF NITROGEN IN TEREDO BARTSCHI CLAPP[1]

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The behavior and morphology of the Teredinidae dictate that the organisms obtain their nourishment either from the wood in which they live as adults, from particulate matter suspended in the water of the respiratory stream, or from a combination of these two sources.

Boynton and Miller (1927) demonstrated the existence of a cellulose-splitting enzyme in *Bankia setacea*, and Lane and Greenfield (1952) have reported the presence of a cellulase associated with the gut of *Teredo*. Previous papers from this laboratory (Lane, Posner and Greenfield, 1952; and Greenfield, 1952) have shown that in adult *T. pedicellata* glycogen may constitute up to 50% of the dry weight, and have suggested that this glycogen reserve could originate from the digestion of the cellulose matrix in which the marine borers live. These observations would appear to support the contention that *Teredo* can employ wood as food. On the contrary, it is a general lamellibranch habit to filter suspended food materials from the water of the respiratory stream, so it might be suspected that planktonic organisms would comprise the chief food supply of *Teredo*.

Greenfield (1953) has reported that the nitrogen content of adult Teredinidae is generally low, the average total nitrogen content of 179 animals being 1.77%. Thus far no work has been done to show the distribution of nitrogen in *Teredo*, nor to identify the raw materials out of which the proteins of the adult borer are constructed.

It is the purpose of this communication briefly to report studies designed to clarify the origin of tissue protein in the Teredinidae.

Materials and Methods

Adult animals to be used for analysis were grown in pine collecting panels measuring 1″ × 6″ × 12″, immersed in Biscayne Bay in a locality known to be infected with shipworms. *Teredo* of useful size for analysis could generally be recovered between six weeks and two months after the original immersion of the wood. The collecting panels were usually completely destroyed by the end of six months.

All the larvae used in the present study were produced in the laboratory. Teredo-infested timbers were maintained in a laboratory tank of circulating sea water. Both supply and overflow lines were protected by 120-mesh Monel screen filters. At intervals of approximately 12 hours the larvae held on the efferent

[1] Contribution No. 99 from the Marine Laboratory, University of Miami. These studies were aided by a contract between the Office of Naval Research and the University of Miami in cooperation with the U. S. Navy Bureau of Yards and Docks.

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filter were washed into a container from which they could be recovered by filtration through paper, or by individual capture with a pipette.

Hydrolyses were effected with 6 N hydrochloric acid, 12 N sulfuric acid or with 10% (w/v) barium hydroxide. After appropriate neutralization and concentration the hydrolysates were chromatographed on Whatman No. 1 paper. Both one- and two-dimensional chromatograms were run to separate various components of the hydrolysates. Each ninhydrin-positive spot originating from the hydrolysates was identified by suitable standard solutions of known amino acids run simultaneously. Solvents employed were water-saturated phenol; the n-butanol, acetic acid, water mixture of Partridge (1948); and water-saturated collidine. Hydrolysis was shown to be incomplete at the end of twenty-four hours by the presence of unresolved peptides. Subsequently all hydrolyses were continued for 48 hours, at which time they were complete.

Wood for analysis was recovered from an uninfected portion of a regular collecting panel. The absence of infection was verified by X-ray examination.

Only complete, intact borers were used for analysis. The size ranged from 1.6 milligrams to 72.8 milligrams dry weight. These figures represent animals between approximately three and 150 millimeters length. Moisture content of adult *Teredo* of different sizes and at different times of the year has proved to average 73%. To determine whether amino acid content or composition differed with growth, the animals were divided into ten arbitrary weight groups with a range of from five to 10 milligrams per group. These groups were always separately analyzed.

The nannoplankton samples hydrolyzed for this study were obtained by filtering sea water through a No. 20 plankton net, discarding the organisms retained by the net, and passing the net-filtered water through an acid-washed asbestos filter pad. The filter and the retained materials were hydrolyzed *in toto*.

Results

Preliminary comparison of acid and basic hydrolysates revealed no qualitative difference in the composition of the amino acid mixture resulting. Subsequent hydrolyses were always conducted in acid media.

The qualitative amino acid content of uninfected regions of wood is compared with the amino acids found in shipworms living in the same panels in Table I.

Table I

Qualitative amino acid content of Teredo and of organic components of its environment

Material hydrolyzed	L	PA	V	T	P	M	A	AR
12-hour larvae	X	0	X	0	X	X	X	X
Teredo less than 50 mg.*	X	X	X	X	X	X	X	X
Teredo more than 50 mg.	X	0	X	X	X	X	X	X
Pine wood	X	0	0	0	0	X	X	X
Nannoplankton	X	0	X	X	X	0	0	0

* Weights are dry-basis.

L—leucine; PA—phenylalanine; V—valine; T—tyrosine; P—proline; M—methionine; A—alanine; AR—arginine.

It will be observed that the wood is deficient in phenylalanine, valine, tyrosine and proline, all of which appear in the hydrolysates of adult shipworms. Hydrolysates of nannoplankton contain all the amino acids which are present in the shipworms but are missing from the wood, with the sole exception of phenylalanine.

The qualitative amino acid content of *Teredo* larvae is also shown in Table I. Larvae differ from the adults qualitatively only in the lack of the aromatic amino acids phenylalanine and tyrosine. It should be emphasized that the amino acids listed in Table I are the only ones found of more than 20 that were sought. Tryptophane, incidentally, has not been detected in any basic or acidic hydrolysate of shipworms of any size.

Discussion

The following experimental considerations are germane to the question of whether the wood excavated during the life of an adult borer—all of which must pass through the digestive system of the animal—could provide all of the nitrogen in the adult worm. The largest *T. bartschi* so far encountered in the exhaustive X-ray examination of more than 100 exposure panels measured 151.5 × 5.9 mm. The volume of this burrow, determined by filling it with paraffin and measuring the displacement of the wax, is most closely approximated by the cone formula. In this instance the maximum volume was 1380 mm.^{3} This figure does not, however, represent the true volume of organic material in the borer. The mantle cavity is filled with water—often maintained under significant pressure (Lane and Tierney, 1951). This water is lost when the borer is removed from the burrow.

A series of fifty worms belonging to this largest size group was weighed. The average, drained weight was 0.350 g. with a moisture content of 73.0%. Total nitrogen (Kjeldahl) varied slightly about the figure of 1.75%. Thus, this largest example of *T. bartschi* contained 0.0016 g. nitrogen.

Wise (1946) states that mature white pine averages 0.3% nitrogen. If the moisture content be assumed to be 10% and the density to be 0.638 g./cm.^{3}, then the wood excavated during the life of this particular borer contained 0.0024 g. of nitrogen.

This would supply the nitrogen actually found in the animal (0.0016 g.) and leave an excess of only 0.0008 g. to supply that which must have been lost by digestive inefficiency, excretion and metabolism during the life of the animal. This may extend from ten to fifteen weeks. It is felt that this excess is inadequate quantitatively. The qualitative inadequacy of wood as a source of nitrogen for *Teredo* has been discussed above. On the contrary a safe excess of nitrogen in the form of amino acids shown to be deficient in wood, is available in nannoplanktonic proteins. It is suggested, therefore, that *Teredo* satisfies its nitrogen requirements by making use both of wood and of suspended planktonic organisms.

Summary and Conclusions

1. The amino acid content of hydrolyzed *Teredo bartschi* Clapp of all ages has been compared chromatographically with hydrolysates of pine wood and of nannoplankton.

2. Wood appears to be deficient in the aromatic amino acids phenylalanine and tyrosine, in the heterocyclic amino acid proline, and in valine, all of which are found in *Teredo*. Hydrolysates of nannoplankton contain all of these missing amino acids except phenylalanine.

3. It is suggested that both wood and suspended nannoplankton are used as dietary sources of nitrogen by *Teredo bartschi*.

LITERATURE CITED

Boynton, L. C., and R. C. Miller, 1927. The occurrence of a cellulase in the shipworm. *J. Biol. Chem.*, **75**: 613–618.

Greenfield, L. J., 1953. Observations on the nitrogen and glycogen content of *Teredo* (*Lyrodus*) *pedicellata* de Quatrefages at Miami, Florida. *Bull. Mar. Sci.*, **2**: 486–496.

Lane, C. E., and J. Q. Tierney, 1951. Hydrodynamics and respiration in *Teredo*. *Bull. Mar. Sci.*, **1**: 104–110.

Lane, Charles E., G. S. Posner and L. J. Greenfield, 1952. The distribution of glycogen in the shipworm, *Teredo* (*Lyrodus*) *pedicellata* Quatrefages. *Bull. Mar. Sci.*, **2**: 385–392.

Lane, Charles E., and L. J. Greenfield, 1952. Physiology of the shipworm. *Amer. J. Physiol.*, **171**: 741.

Partridge, S. M., 1948. Filter paper partition chromatography of sugars. *Biochem. J.*, **42**: 238–250.

Wise, L. E., 1946. Wood chemistry. Rheinhold Publishing Company, New York.

THE PHYSIOLOGY OF INSECT DIAPAUSE. VII. THE RESPIRATORY METABOLISM OF THE CECROPIA SILKWORM DURING DIAPAUSE AND DEVELOPMENT[1]

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The endocrine control of the pupal diapause of the Cecropia silkworm has been the principal theme of the preceding papers of this series. A considerable body of evidence is now at hand pointing to the prothoracic glands as the source of a hormone which reacts with the tissues to terminate the pupal diapause. The prothoracic gland hormone, in turn, has been identified as the "moulting hormone," the "pupation hormone," and the "growth and differentiation hormone" of previous investigators (*cf.* Williams, 1952).

The present paper initiates a series of reports in which the analysis is narrowed to the reaction of the hormone with the pupal tissues. The central though necessarily distant objective is to define the biochemical events which couple the action of the hormone to the biological end-results. It seemed possible that this elusive goal might be particularly accessible in the case of the diapausing insect where the prothoracic gland hormone produces prompt and spectacular effects in each and every tissue. As a first step in this direction the present study considers the respiratory metabolism of the Cecropia silkworm during diapause and development.

Materials and Methods

1. *Experimental animals*

The present investigation is based on approximately 1500 respiratory measurements performed on a total of 150 larvae, pupae, and developing adults of the giant silkworm *Platysamia cecropia*. One series of experiments was carried out on pupae of the closely related Cynthia silkworm (*Samia walkeri*). The insects were reared from eggs and managed according to techniques described previously (Williams, 1946).

2. *Apparatus*

Most of the measurements were performed at 25° C. in 45-cc. vessels equipped with venting-plugs and adapters for use with standard Warburg manometers (Fig. 1). In occasional experiments the gas volume of the system was decreased by the insertion of lucite "space-occupiers" into one or more vessels. Certain preliminary experiments were carried out in 25-cc. non-compensated capillary respirometers.

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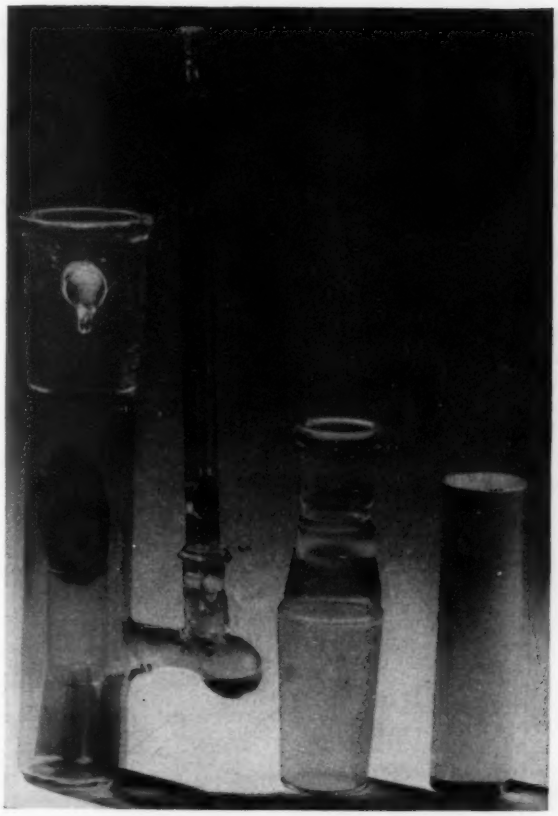

FIGURE 1. Respirometer vessel for use with standard Warburg manometer. The vessel is equipped with a side-arm, a venting-plug, and an adapter for junction with the manometer. The pupa is supported on a glass tube above alkali-moistened filter paper. The lucite cylinder on the extreme right is a space occupier for increasing the sensitivity of the system.

The carbon dioxide output was absorbed on a strip of filter paper moistened with 0.3 to 0.5 cc. of 1.5 N sodium hydroxide. Measurements were performed over periods that averaged twenty hours, the excursions of the manometer being recorded at intervals of from five minutes to eight hours, as dictated by the rate of oxygen uptake.

At the conclusion of each experiment, acid was added to displace the carbon dioxide from the alkali, and the volume of the evolved gas determined in an analyzer of the type described by Bliss (1953). The several precautions outlined by Scholander *et al.* (1952) for experiments of this type were observed. The total output of carbon dioxide was determined from the moment the vessels were sealed until the end of the experiment, and the average calculated over this period. The

over-all respiratory quotient of each insect was calculated by dividing the average rate of carbon dioxide output by the average rate of oxygen consumption.

Oxygen Consumption During Metamorphosis

Enormous changes were observed in the rate of oxygen consumption of the Cecropia silkworm during the course of its metamorphosis at 25° C. In Figure 2 these changes have been assembled and plotted, the discontinuity in the x-axis corresponding to the several months of temporary storage at 5° C. prior to the

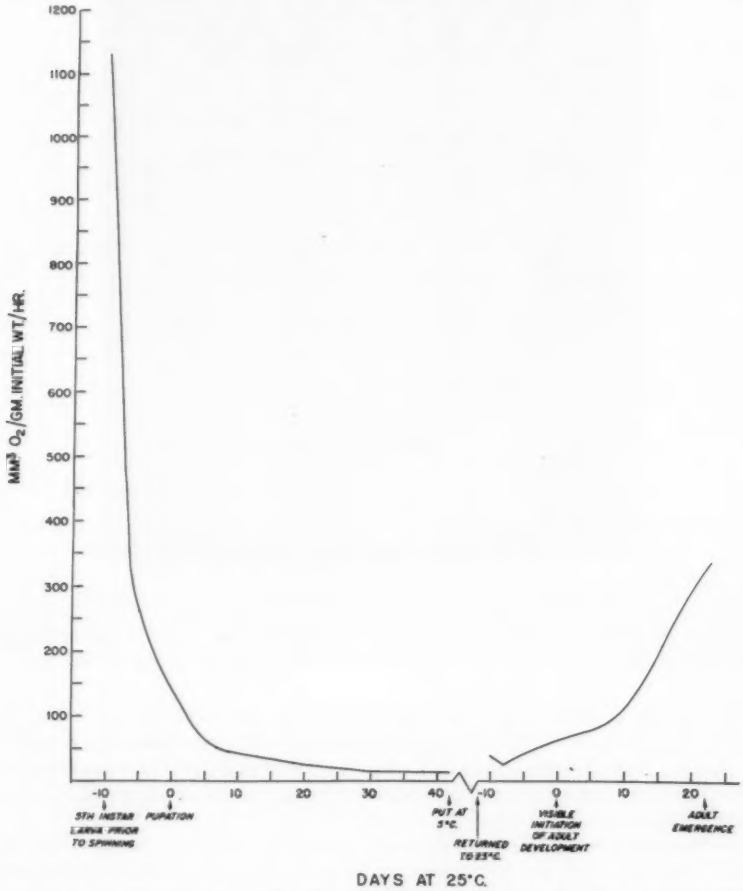

Figure 2. The average rate of oxygen consumption at 25° C. of the Cecropia silkworm during its life history. The rates of oxygen consumption are expressed as mm³. oxygen per gram initial weight per hour; the "initial weight" refers to the weight of each individual two to three weeks after pupation.

return of the diapausing pupae to 25° C. In each instance the rates of oxygen consumption of the various animals have been computed in terms of the live weight of each individual several weeks after pupation. This procedure, the use of the pupal weights in the computations, automatically compensated for the large weight loss of the mature larva prior to pupation and of the pupa during the course of adult development.

The several segments of the curve will now be considered in further detail.

1. Larva, prepupa, and pupa

Figure 3 illustrates the precipitous decrease in the rate of oxygen consumption of Cecropia during the larval-pupal transformation. Similar measurements are recorded in Figure 4 for the closely related Cynthia silkworm. The animals were maintained at 25° C. and all measurements performed at this temperature.

It is clear that a striking decrease in the insect's metabolism begins just after the cocoon has been spun and continues throughout the prepupal and early pupal periods. By the second week after pupation the rate of oxygen consumption, in the case of Cecropia, is 2.5 per cent of that of the mature larva. By about the third week following pupation the rate of oxygen consumption reaches an extremely low level where it persists during the many months of pupal diapause at 25° C.

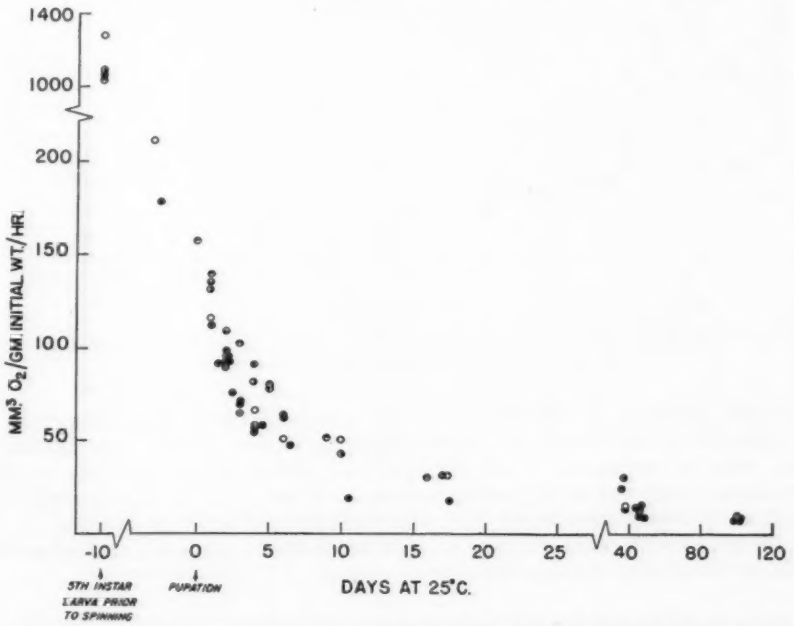

FIGURE 3. The rate of oxygen consumption of mature fifth instar Cecropia larvae at 25° C. just prior to spinning (ten days before pupation) and at frequent intervals thereafter. The measurements on larvae are determinations during periods when the animals were quiescent within the respirometers.

Ten to sixteen weeks after pupation, 43 diapausing Cecropia pupae, varying in weight from 4 to 6 gm., showed an average oxygen consumption of 16.3 ± 9.03 (s.d.)mm.^{3} per gram live weight per hour. The median was 13.7 and the extremes 6.8 to 38.0. On a dry weight basis the average was 65.0 mm.^{3} per gram per hour. These values are but 1.4 per cent of the corresponding measurements of mature larvae and 11 per cent of those of fresh pupae.

2. Previously chilled pupae prior to the initiation of adult development

Figure 5 records the respiration of five previously chilled pupae at frequent intervals after being returned to 25° C. The first visible signs of adult development

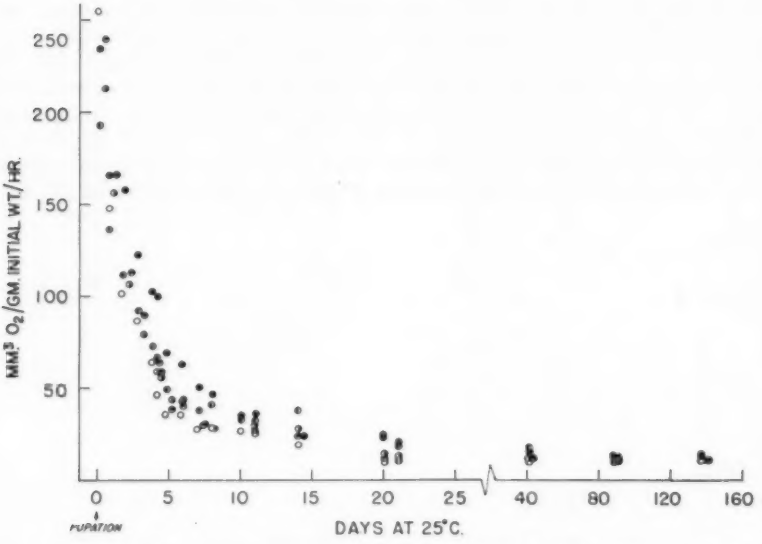

FIGURE 4. The rates of oxygen consumption at 25° C. of five Cynthia silkworms at the time of pupation and at frequent intervals thereafter.

(Williams, 1946) appeared after an average of 13 days at the high temperature. The behavior of these previously chilled diapausing pupae illustrates two significant facts—that the metabolism is still extremely low after prolonged exposure to low temperature, and that a progressive increase in metabolism becomes evident approximately a week prior to the first visible indication of adult development.

Identical results were obtained in a second series of previously chilled pupae which initiated adult development after an average of 31 days at 25° C.

3. After the initiation of adult development

Figure 6 records the characteristic changes observed in the rate of oxygen consumption of several previously chilled pupae during the course of adult development

at 25° C. The results are typical of a number of experiments of this type. The initiation of adult development is characterized by a continuation of the increasing metabolism which, as we have just seen, had already been evident a week before the visible initiation of development. During the final two-thirds of adult develop-

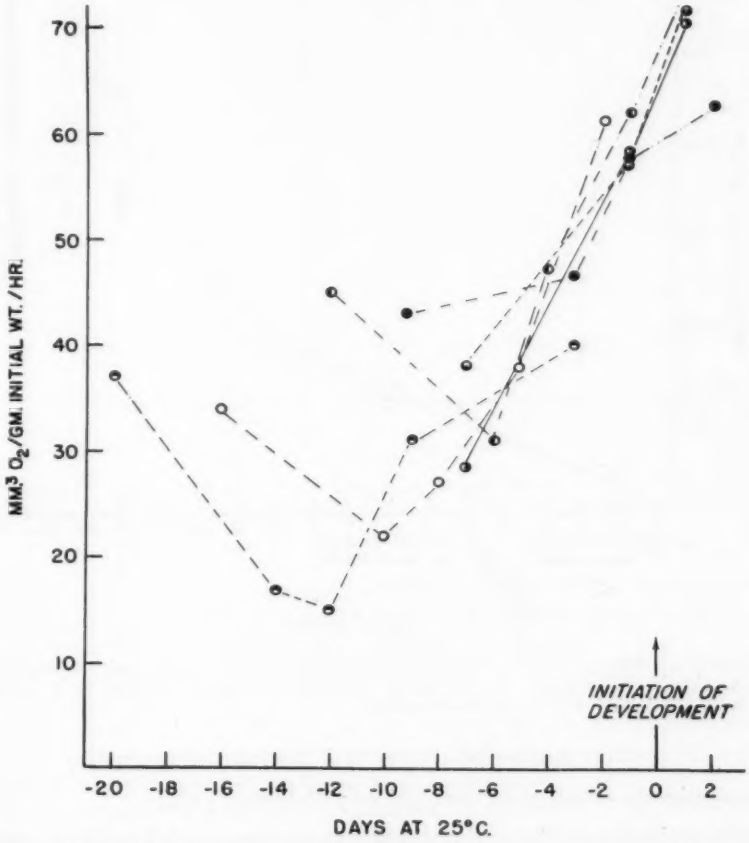

FIGURE 5. Rates of oxygen consumption of five previously chilled pupae after return to 25° C. The initial measurements for each animal were made two days after removal from the low temperature. The respiratory metabolism begins to increase about one week prior to the visible initiation of adult development.

ment, the rate of oxygen consumption increases yet more rapidly. Just prior to emergence, the average respiration of five fully formed adult moths was 312 mm.³ of oxygen per gram initial live pupal weight per hour. This value is 19 times that of diapausing pupae, but only ⅓ that of mature larvae.

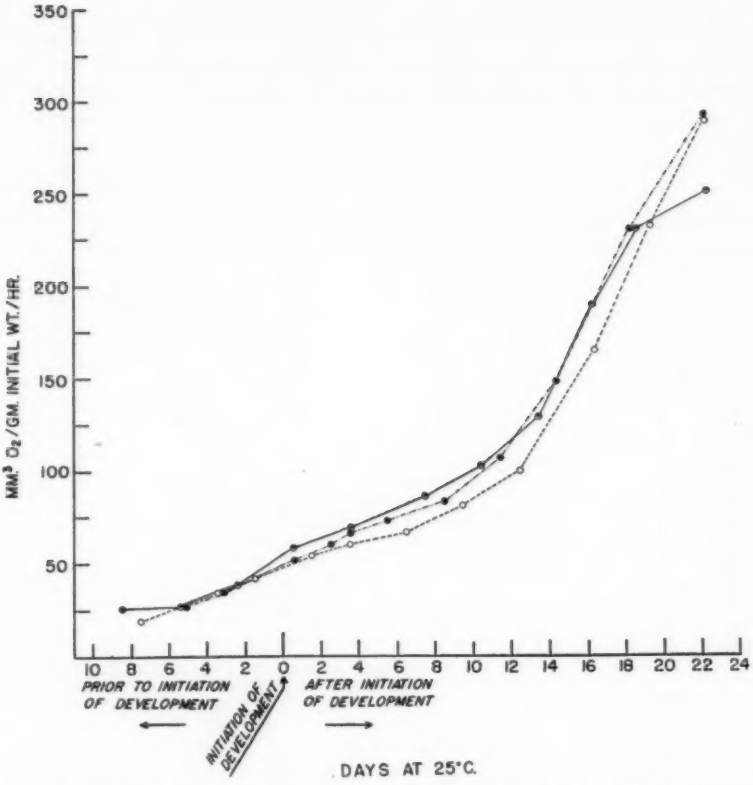

FIGURE 6. Rates of oxygen consumption during the course of the adult development of three previously chilled pupae. In this group of animals the adults emerged 24 days (average) after the initiation of adult development.

THE RESPIRATORY QUOTIENT DURING DIAPAUSE

1. Discontinuous release of carbon dioxide by diapausing pupae

The respiratory quotients of diapausing pupae were studied at 25° C. in animals previously stored for several months at this same temperature. In preliminary experiments an astonishing degree of scatter was encountered in the respiratory quotients when the latter were determined over one-hour intervals. Individual animals showed quotients which varied from 0.1 to 3.0 during successive hours—a result which suggested that either the intake of oxygen and/or the release of carbon dioxide were discontinuous.

Though a detailed study of the matter will be considered in a subsequent communication, we can state that experiments testing these possibilities have provided results of the type illustrated in Figure 7. Whereas the rate of oxygen utilization

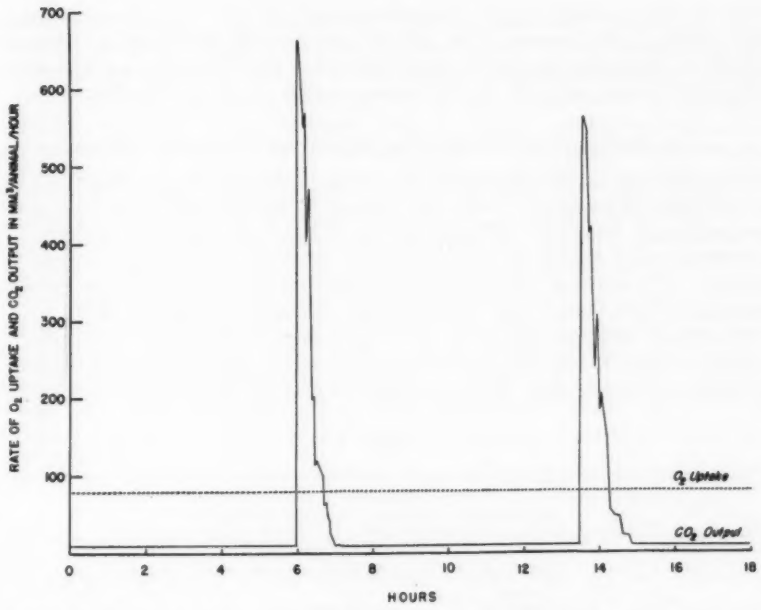

FIGURE 7. Rates of oxygen consumption and carbon dioxide output of a diapausing Cecropia pupa at 25° C. over an eighteen-hour interval. The "direct method" of Warburg was employed in this experiment.

was essentially constant, an extraordinary degree of discontinuity was observed in the rate of output of carbon dioxide. The latter was "blown off" in "bursts" every few hours, less than 10 per cent being excreted continuously.

We have consistently observed this phenomenon in all diapausing lepidopterous pupae examined—a finding in substantial agreement with the detailed results reported by Punt (1948, 1950). For our present purposes the discontinuous release of carbon dioxide is of interest because it illustrates a serious problem in the estimation of the respiratory quotients of diapausing pupae. It is clear that only prolonged measurements can provide a reasonable indication of the ratio between carbon dioxide release and oxygen consumption.

2. *Respiratory quotient of diapausing pupae*

The respiratory quotients of 49 diapausing Cecropia pupae were determined at 25° C. after three to five months of storage at this same temperature. Each experiment was continued for at least 17 hours in order to encompass two or more carbon dioxide bursts. The average respiratory quotient was 0.78 ± 0.093 (s.d.); the medium was 0.77 with extremes of 0.57 to 1.00. A similar series of determinations on seven brainless diapausing pupae recorded an average respiratory

quotient of 0.72 ± 0.091 (s.d.); the median was 0.72 with extremes of 0.57 to 0.87. Consequently, there appears to be nothing peculiar about the average respiratory quotient of diapausing Cecropia pupae, provided that the experimental technique is adequate to cope with the discontinuous release of carbon dioxide.

Stimulation of Pupal Metabolism by Localized Injury

In experiments testing the metabolic effects of various drugs when injected into diapausing Cecropia pupae, a prompt increase in the rate of oxygen consumption was consistently observed. It soon became evident that the effect was due, not to the injected drug, but to the perforation of the integument in the course of the injection. For example, the same result was induced by puncturing the integument with a dry needle. The phenomenon was studied in further detail.

The effects of minor injury to the integument are illustrated in the data summarized in Table I. In this experiment the average rates of oxygen consumption and carbon dioxide output were first measured at 25° C. in a series of nine dia-

Table I

Effects of small integumentary incisions on the respiration of diapausing Cecropia pupae at 25° C. The anti-tyrosinase, phenylthiourea, was implanted into three of the nine individuals at the time of incision (Group B)

Experimental time	Group A No PTU when incised (6 animals)		Group B Received PTU when incised (3 animals)		Over-all average (9 animals)	
	Relative rate of oxygen consumption	R. Q.	Relative rate of oxygen consumption	R. Q.	Relative rate of oxygen consumption	R. Q.
1 day prior to incision	100%	.81	100%	.88	100%	.84±.13 (s.d.)
1 day after incision	272%	.81	313%	.81	287±59 (s.d.) %	.81±.06 (s.d.)
6 days after incision	183%	.84	193%	.72	186±42 (s.d.) %	.80±.09 (s.d.)
22 days after incision	77%	—	81%	—	78±16 (s.d.) %	—

pausing Cecropia pupae. A V-shaped incision having a total length of 4 mm. was then made in the thoracic tergum of each individual just lateral to the mid-line. Into three of the animals crystals of the anti-tyrosinase, phenylthiourea, were implanted at this time. The sites of injury were then sealed with melted paraffin. Twenty-four hour measurements of the rates of oxygen consumption and carbon dioxide output were then performed every few days.

The rate of oxygen consumption of each individual began to increase within one hour and became maximal within twenty-four hours; at this time it averaged 287 ± 59 (s.d.) per cent of the pre-injury values (extremes of 224 and 424 per cent). Six days after the injury the rates of oxygen consumption were still elevated at 186 ± 42 (s.d.) per cent of the initial values. By the twenty-second day the injury metabolism had totally disappeared and the rate of oxygen consumption had returned to 78 ± 16 (s.d.) per cent of its value prior to injury.

Table I also indicates that the potent anti-tyrosinase, phenylthiourea, was with-

out effect in opposing or preventing the injury metabolism. Moreover, since the respiratory quotient remained unchanged after injury, it is clear that the oxygen consumption and carbon dioxide production were stimulated in the same proportion.

The preceding experiments were performed on pupae capable of moving their abdominal segments. It seemed possible that the injury metabolism had its source in increased activity of the abdominal musculature. Two lines of experimentation were performed to test this possibility. In the first of these, the effects of injury were studied on isolated anterior and posterior ends of diapausing pupae. The injury metabolism was found to be just as pronounced in the immotile anterior fragments as in the motile posterior fragments. In the second type of experiment, a series of four pupae was immobilized by the removal of the entire chain of abdominal ganglia and connectives, thereby denervating the intersegmental muscles of the abdomen. Two months later the rates of oxygen consumption were measured, following which the thoracic tergum of each individual was punctured with a dry 22-gauge hypodermic needle inserted to a depth of 4 mm. Two hours after the injury the rates of oxygen consumption showed an average increase of 42 ± 17.5 (s.d.) per cent. It is clear that muscular activity is not responsible for the injury metabolism.

TABLE II

Effects of extensive injury to the integument on the respiration of nine diapausing Cecropia pupae at 25° C.*

Experimental time	Relative rate of oxygen consumption		
	Average	Median	Range
1 day prior to operation	100%	96%	69–145%
3 days after operation	814%	750%	570–1450%
69 days after operation	291%	207%	101–716%

* The integument overlying the pupal legs was removed and replaced by a triangular plastic slip sealed in place with melted paraffin. Crystals of phenylthiourea were implanted into each animal at the time of injury.

The magnitude and persistence of the injury metabolism appeared to vary with the extent of the injury. Major surgical procedures caused a tremendous increase in the rate of oxygen consumption which persisted for long periods thereafter. This fact is illustrated in the experiments summarized in Table II.

It is of particular interest and importance that diapausing pupae showed no over-all morphological change in response to the injury metabolism. Even when the metabolism was increased as high as 14-fold, the pupae continued to diapause—a fact confirmed by day-to-day observations and by dissections at the conclusion of the experiments.

DISCUSSION

Just prior to the spinning of the cocoon the mature fifth instar Cecropia larva ceases the intake of substances other than atmospheric oxygen. Henceforth until its death as a mature moth ten months later, the insect reworks according to its needs the materials of its own body. The larva, whose metabolism per unit mass

ranks among the highest recorded for non-flying invertebrates, transforms into a diapausing pupa, whose metabolism is among the lowest noted for any animal (*cf.* Robbie, 1949; Heilbrunn, 1952). Then, months later, yet another transformation converts the diapausing pupa into the adult moth—a flying machine whose metabolism during flight is approximately two thousand times that of the pupa.

These results (Fig. 2) are in substantial agreement with those described by Heller (1926) for the diapausing sphingid, *Deilephila*. Like Heller, we observe that the months of pupal diapause are characterized by an extremely low metabolic rate, corresponding to the horizontal portion of the U-shaped curve of metabolism.

It is not our present purpose to consider the significance of the U-shaped curve in detail, but only to call attention to the metabolic characteristics of the two great periods of rapid rearrangement and maximal morphogenetic change. During the first of these, the prepupal period, the respiratory metabolism *decreases* more precipitously than at any other stage in the life history. During the second period of rapid change, the first few days of adult development, the over-all respiratory metabolism is extremely low—lower, in fact, than that which one can induce by injury to pupae during diapause. It is indeed true, as indicated in Figure 2, that the overall metabolism undergoes considerable increase during the latter stages of adult development. But this change mirrors the construction and maintenance of the future flying machine and, in our opinion, has no direct bearing on the earlier events which terminate the pupal diapause.

For these several reasons we are unimpressed by arguments which emphasize the high metabolic cost of morphogenesis and attribute the state of diapause to a simple quantitative deficiency in the over-all respiratory metabolism.

All of our experiments indicate that the oxygen consumption begins gradually to increase about one week prior to the visible initiation of adult development. Boell (1935) records a similar observation on diapausing grasshopper embryos where changes in respiratory rate were detected two days before the development of the embryo became apparent. This increment of respiration apparently signals a change in the pre-existing steady state of diapause and, in the case of Cecropia, suggests that the biochemical processes responsible for the initiation of adult development are set in motion about a week before one can detect any morphological evidence of the termination of diapause. The visible initiation of development is evidently the end-product of a brief period of endocrinological and biochemical preparation. Since the onset of adult development is brought to pass by the reaction between the growth and differentiation hormone and the diapausing tissues, the respiratory measurements suggest that the secretion of this hormone at 25° C. begins about a week before the actual initiation of adult development.

Turning to a consideration of the respiratory quotient, the measurements on diapausing Cecropia reveal nothing unusual, aside from the fact that the carbon dioxide is given off intermittently. A total of 49 determinations based on prolonged measurements showed an average respiratory quotient of 0.78 ± 0.093 (s.d.)—a value consistent with the combustion of a mixture of fat, protein, and carbohydrate. Though the respiratory quotients of diapausing lepidopterous pupae have hitherto been studied only by Agrell (1947, 1951a), the above-mentioned value is in good agreement with numerous determinations previously reported for non-diapausing pupae and for the changes occurring in the corresponding substrates (for summary see Needham, 1942, and Portier, 1949).

Our findings on Cecropia therefore stand in marked contrast to those described by Agrell (1947, 1951a) for the diapausing lepidopterous pupae, *Phalera* and *Endromis*. In measurements performed at 18° C. Agrell recorded respiratory quotients ranging from 0.1 to 0.3. On the basis of these extremely low values, Agrell has proposed a comprehensive theory of diapause (1951a, 1951b). According to this theory, the enzymes serving decarboxylation are defective in diapausing pupae due to a deficiency in thiamine and other vitamins acting as prosthetic groups in decarboxylases. The hormone which terminates diapause does so, he suggests, by promoting the synthesis of these particular vitamins, thereby reconstituting the decarboxylases. Previously thereto, the low respiratory quotients of diapausing pupae signal large scale conversion of fats into carbohydrates.

The respiratory quotients which formed the factual basis of this theory were determined by Agrell using the Thunberg microrespirometer. Each respiratory quotient was based on measurements over three to five successive periods of about one hour each. Agrell reports that the respiratory quotients obtained during these successive periods were uniform—a result indicative of a steady consumption of oxygen and release of carbon dioxide. But in our experience, as well as in that of Punt (1948, 1950), the carbon dioxide output of all the diapausing lepidopterous pupae examined (Sphingidae, Saturniidae, Noctuidae, Papilionidae) is characterized by singular discontinuity. In Cecropia at 25° C. less than 10 per cent of the carbon dioxide is released continuously. The remaining 90 + per cent is stored for periods averaging eight hours and then released within a one hour period (unpublished observations).

Though Agrell (1951a) suggests that the low respiratory quotients which he reports had (p. 283) "nothing to do with the rhythmic expulsion of carbon dioxide," it is difficult to be certain of this fact. Unless *Phalera* and *Endromis* differ from other genera and species of diapausing Lepidoptera, the release of carbon dioxide occurs during short, widely spaced intervals rarely encompassed within brief periods of measurement. In these same terms the increase in the respiratory quotient which Agrell observed at the outset of adult development could be accounted for, since the release of carbon dioxide is known to be less discontinuous after adult development has been initiated (Punt, 1948, 1950) and a representative sample could probably be obtained during brief measurements.

Since the determinations of Cecropia were performed over periods of not less than 17 hours, we conclude that the average respiratory quotient of 0.78 is a valid indication of the type of metabolism of diapausing Cecropia pupae, and that it probably signifies the combustion of a mixture of fats, proteins, and carbohydrates. Agrell's theory of diapause therefore appears to be unacceptable in the case of the Cecropia silkworm.

The injury-stimulated metabolism of Cecropia pupae is a phenomenon no less curious than the discontinuous release of carbon dioxide. A simple perforation of the integument reacts on the animal as a whole to cause a prompt and pronounced increase in the rates of oxygen consumption and carbon dioxide production. In the case of more extensive injury, the metabolism increases as much as 14-fold and requires longer than 2½ months to return to normal.

These results have certain parallels in the literature. Agrell (1951a) reports

that the injection of physiological saline caused an 80 per cent increase in the respiration of diapausing pupae of *Endromis*, while Sussman (1952) has previously noted the phenomenon in the case of Cecropia. In *Calliphora* adults, injury is without effects on oxygen consumption (Thomsen, 1949); in *Periplaneta* adults, the injury-stimulated respiration persists for only 5 hours (Hassett, 1948); in the unfertilized eggs of *Bombyx*, only 24 hours (Wolsky, 1939). In this connection the large and prolonged changes in the colligative properties of the blood which Robinson (1928) observed after integumentary injury to diapausing pupae of the giant silkworm, *Telea polyphemus*, are of special interest.

The injury metabolism, as we have seen, is characterized by a proportional increase in both oxygen consumption and carbon dioxide output. The effect is not a by-product of increased muscular activity, since it persists in fragments devoid of somatic musculature and in animals in which the muscles have been denervated. Finally, the increased metabolism is insensitive to phenylthiourea and, consequently, is not mediated by tyrosinase.

Though the basis of the injury-stimulated respiration remains undefined, it is clear that the metabolism which it mirrors cannot be equated to the metabolism of the developing post-diapausing insect. For, though the rate of respiration of an injured diapausing pupa can increase to a level characteristic of animals halfway through adult development, no development occurs. Thus, the diapausing pupa, while capable of respiring at rates that characterize the growing insect, fails to grow in the absence of the proper hormonal stimulus and the proper *quality* of metabolism. It would appear that the increment of respiration induced by injury cannot be coupled to morphogenesis.

The injury-stimulated respiration, in itself, gives assurance that the absence of morphogenesis during diapause is not attributable to a simple *quantitative* deficiency in the dehydrogenase enzymes which release hydrogen from substrate, or in the redox enzymes which transmit the hydrogen to oxygen.

We wish to thank Mr. Dietrich Bodenstein and Dr. Leigh E. Chadwick for critical reading of the manuscript of the present paper.

Summary

1. The over-all metabolism of the Cecropia silkworm, as signaled by its respiratory exchange, undergoes pronounced alterations during the course of the larval-pupal-adult transformation.

2. The rate of oxygen consumption of the diapausing pupa, averaging at 25° C. 16.3 ± 9.03 (s.d.) $mm.^3$ per gram live weight per hour, is only 1.4 per cent that of the mature larva and 5 per cent that of the adult moth just prior to ecdysis. The metabolism at 25° C. persists at or near this low level throughout the pupal diapause.

3. One week prior to the termination of diapause the metabolism begins gradually to increase. This change apparently accompanies the initiation of the endocrine events which culminate in the termination of the pupal diapause. By the first day of visible adult development the rate of oxygen consumption is 3½ times that of the diapausing pupa.

4. The continuation of adult development after the termination of diapause is accompanied by further increase in the rate of respiratory exchange—a change which mirrors the differentiation of the adult moth.

5. It was found that localized perforations or other injuries to the integument of diapausing pupae caused a pronounced increase in the insect's metabolism. The magnitude and persistence of the enhanced respiration varied with the extent of the initial injury. After extensive injury, the metabolism increased up to 14-fold and required over 2½ months to return to initial levels. The injury metabolism was unaffected by the anti-tyrosinase, phenylthiourea.

6. Though the metabolism of diapausing pupa could in this manner be increased to levels characteristic of the post-diapausing insect mid-way in adult development, no development took place. The significance of this finding is considered in relation to the biochemical basis of diapause.

7. It was found that whereas the utilization of oxygen by diapausing pupae is continuous, the release of carbon dioxide is discontinuous. Ninety per cent of the carbon dioxide is accumulated and "blown off" in short "bursts" which, at 25° C., occur about once every eight hours. This fact necessitated prolonged experiments for the determination of respiratory quotients.

8. In experiments averaging 20 hours in duration, 49 diapausing Cecropia pupae showed an average respiratory quotient of 0.78 ± 0.093 (s.d)—a value consistent with the combustion of a mixture of fats, carbohydrates, and proteins during diapause.

9. No indication was found of the extremely low average respiratory quotients, the multiple deficiencies in vitamins serving decarboxylation, or the conversion metabolism which Agrell has postulated as the biochemical basis of the pupal diapause.

LITERATURE CITED

AGRELL, I., 1947. Some experiments concerning thermal adjustment and respiratory metabolism in insects. *Arkiv för Zoologi*, **39**: 1–48.

AGRELL, I., 1951a. Pupal diapause caused by a vitamin deficiency. *Nature*, **167**: 283.

AGRELL, I., 1951b. The diapause problem. *Année Biologique*, **27**: 287–295.

BLISS, D., 1953. Endocrine control of metabolism in the land crab, *Gecarcinus lateralis* (Fréminville). I. Differences in the respiratory metabolism of sinusglandless and eyestalkless crabs. *Biol. Bull.*, **104**: 275–296.

HASSETT, C. C., 1948. Effect of ryanodine on the oxygen consumption of *Periplaneta americana*. *Science*, **108**: 138–139.

HEILBRUNN, L. V., 1952. An outline of general physiology. Third Ed. W. B. Saunders and Co., Philadelphia.

HELLER, J., 1926. Chemische Untersuchungen über die Metamorphose der Insekten III. Über die "subitane" und "latente" Entwicklung. *Biochem. Zeitschr.*, **169**: 208–234.

NEEDHAM, J., 1942. Biochemistry and morphogenesis. Cambridge University Press.

PORTIER, P., 1949. La biologie des Lépidoptères. *Encyclopédie Entomologique, A*, **23**. Paris.

PUNT, A., 1948. The respiration of insects during hibernation. *Acta Brevia Neerlandica*, **16**: 30.

PUNT, A., 1950. The respiration of insects. *Physiol. Comp. et Oecol.*, **2**: 59–74.

ROBBIE, W. A., 1949. Respiration of the tissues of some invertebrates and its inhibition by cyanide. *J. Gen. Physiol.*, **32**: 655–670.

ROBINSON, W., 1928. A study of the effect of surgical shock on insects. *J. Agric. Res.*, **37**: 743–755.

SCHOLANDER, P. F., C. L. CLAFF, J. R. ANDREWS AND D. F. WALLACH, 1952. Microvolumetric respirometry. *J. Gen. Physiol.*, **35**: 375–395.

SUSSMAN, A. D., 1952. Tyrosinase and the respiration of pupae of *Platysamia cecropia* L. *Biol. Bull.*, **102**: 39–47.

THOMSEN, E., 1949. Influence of the corpus allatum on the oxygen consumption of adult *Calliphora erythrocephala* Meig. *J. Exp. Biol.*, **26**: 137–149.

WILLIAMS, C. M., 1946. Physiology of insect diapause: the role of the brain in the production and termination of pupal dormancy in the giant silkworm, *Platysamia cecropia*. *Biol. Bull.*, **90**: 234–243.

WILLIAMS, C. M., 1952. Morphogenesis and the metamorphosis of insects. *Harvey Lectures*, **47**: 126–155.

WOLSKY, A., 1939. The respiratory mechanism of silkworm eggs. II. The oxygen consumption of damaged sterile eggs. *Magyar Biol. Kutato intezet Munkai*, **11**: 375–381. (*Chem. Abstracts*, 1940).

THE DISTRIBUTION OF THE POLYCHAETE NEANTHES LIGHTI IN THE SALINAS RIVER ESTUARY, CALIFORNIA, IN RELATION TO SALINITY, 1948–1952

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The estuarine nereids, with representatives widely distributed, offer excellent material for studies in comparative physiology and ecology. The present study is part of an attempt to work out the nature of the adaptation to brackish waters as seen in one species occupying ecologically distinctive habitats within a limited geographical area. *Neanthes lighti* furnishes favorable material for studies of the invasion of fresh waters by marine annelids, since it equals or exceeds the much-studied *Nereis diversicolor* in its ability to live and reproduce in waters of low salinity. Its viviparous mode of reproduction, described in an earlier report (Smith, 1950), has perhaps been of adaptive significance in this respect. The fact that the species appears to consist of self-fertilizing hermaphroditic individuals, living in widely separated rivers, may have favored the appearance of local races, reproductively and spatially isolated from each other. In the Salinas River estuary, some twelve miles north of Monterey, California, the species occurs under a wide range of conditions, and so furnishes material for a study of ecological limitations and adaptations which can form a background for, and give meaning to, studies upon its physiology. As a preliminary to more detailed studies upon the mechanism of osmotic regulation, the present report describes the physical and biotic conditions under which *N. lighti* occurs in the Salinas River. These findings in themselves depict extremely variable estuarine conditions, and may be useful in studies on other animals, and in amplifying our information on estuaries in general. An understanding of the history of salinity changes in an estuary is a prerequisite for studies on the distribution and physiology of its inhabitants.

Observations were begun in 1948, coincidentally with other work, and have been carried out as opportunity offered until the fall of 1952. During certain periods the river could be visited only infrequently, hence the data include numerous gaps. However, the total four and one half year record does reveal in a general way the pattern of salinity changes in an estuarine system, and covers the full range of climatic conditions characteristic of this area. It is believed that the results clarify the relationship of salinity to climatic conditions, and will make future studies of estuaries in this area more rewarding.

Physical and Biotic Conditions in the Salinas Estuary

The estuary of the Salinas River (Fig. 1) presents an extremely varied set of conditions, especially in relation to salinity. This variation in salinity characterizes estuaries in general; for an excellent discussion of estuarine conditions, the review by Day (1951) should be consulted. The Salinas falls into Day's category

of "blind" estuaries,[1] that is to say, it carries in most parts of the year so small a flow that across its mouth the sea builds up a barrier sand bar, blocking normal outflow and tidal exchange. When this has occurred, the estuary gradually freshens, often standing at a high level. In winter, flood conditions may occur

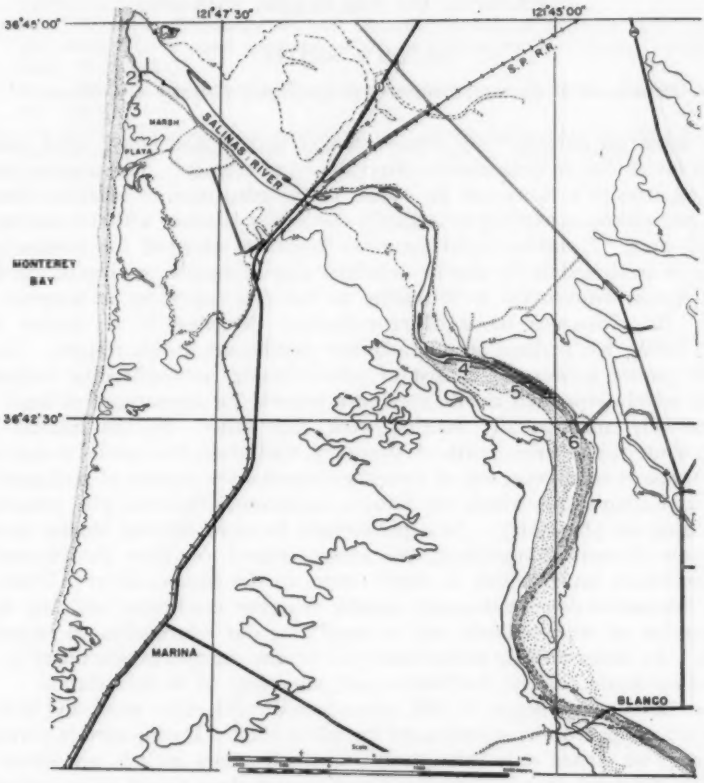

FIGURE 1. The estuary of the Salinas River, from Army Map Service sheets (Marina and Salinas quadrangles) compiled in 1947, to show locations 1–7 mentioned in text. Most of detail omitted; only 10, 20 and 100 foot contours shown.

following periods of heavy rain. In most years these seasonal freshets have caused the river to cut through the bar across its mouth, occasionally in the past rather violently, and with loss of life (MacGinitie, 1935), providing for a time an outlet to the sea. In recent years it has been the practice to excavate a channel through the barrier of sand in order to release the swollen river before flooding of farmlands oc-

[1] Judging from the account given by Scott, Harrison and Macnae (1952), the Klein River Estuary in South Africa bears a close general resemblance to the Salinas Estuary.

curs. With the breaching of the barrier, the lower and middle reaches of the estuary receive tidal influxes of sea water until the bar is reëstablished. Thus the lower reaches for most of the year experience low salinity but are seasonally subjected to extreme fluctuations in salinity and exposure. The upper reaches of the estuary and marshes adjacent to the lower reaches also undergo salinity fluctuations, but not nearly so drastic in magnitude and rate of change. Were seasonal rainfall and the consequent fresh water discharge into the estuary uniform from year to year, a description of salinity conditions would be relatively simple, but in fact no such uniformity exists. In order to provide a background for understanding the changes in the river during the years 1948–52 of this study, rainfall and runoff records since 1944 have been compiled from published records (U. S. Depts. of the Interior and of Commerce). As shown in Figure 2, practically all of the rainfall in this region falls between September and April, and even within this span of months may be extremely concentrated, sending great freshets down into the estuary in certain winter months. The watershed of the Salinas is stated to be 4231 square miles, and rainfall varies considerably in different parts of the area. In order to make a rough estimate of the rainfall, the monthly rainfalls in inches reported from all

TABLE I

Total runoff in "second-foot-days"[2] entering Salinas Estuary by water-years (Oct.–Sept.), and totaled average rainfall at stations recording on Salinas River watershed

Water-year	Rainfall (inches)	Runoff
1944–45	12.11	157,147
1945–46	12.41	66,692
1946–47	9.25	3,513
1947–48	11.24	1,645
1948–49	11.56	25,500
1949–50	12.35	14,874
1950–51	10.21	17,862
1951–52	20.53	336,957

stations on the watershed (4 to 8 stations in most months) were averaged, and the sum of the monthly rainfalls taken as an expression of total rainfall for each water year (October through the following September). These estimates showed a more than two-fold variation in annual rainfall, from about 10 inches in the dry years 1946–48, to 20 inches in the extremely wet 1951–52 season (Table I).

Discharge into the estuary has been taken from the records of the gauging station at Spreckels, a few miles upstream, near the city of Salinas. These values show a far more extreme variation than do rainfall records, the total yearly discharge varying from 1645 second-foot-days[2] (142,128,000 cu. ft.) in 1947–48 to 336,957 second-foot-days (29,113,084,800 cu. ft.) in 1951–52 (Table I and Fig. 2). Impoundments and irrigation diversions undoubtedly greatly reduced the discharge in the years of low rainfall, and may have contributed materially to the stability which characterized the estuary prior to 1949. Variation from month to month is also extreme, the record of 173,901 second-foot-days in the month of February, 1952 exceeding the combined discharge of the six preceding water-years.

[2] One "second-foot-day" is the amount of water flowing in one day past a point at a rate of one cubic foot per second. It equals 86,400 cubic feet or 1.983 acre-feet.

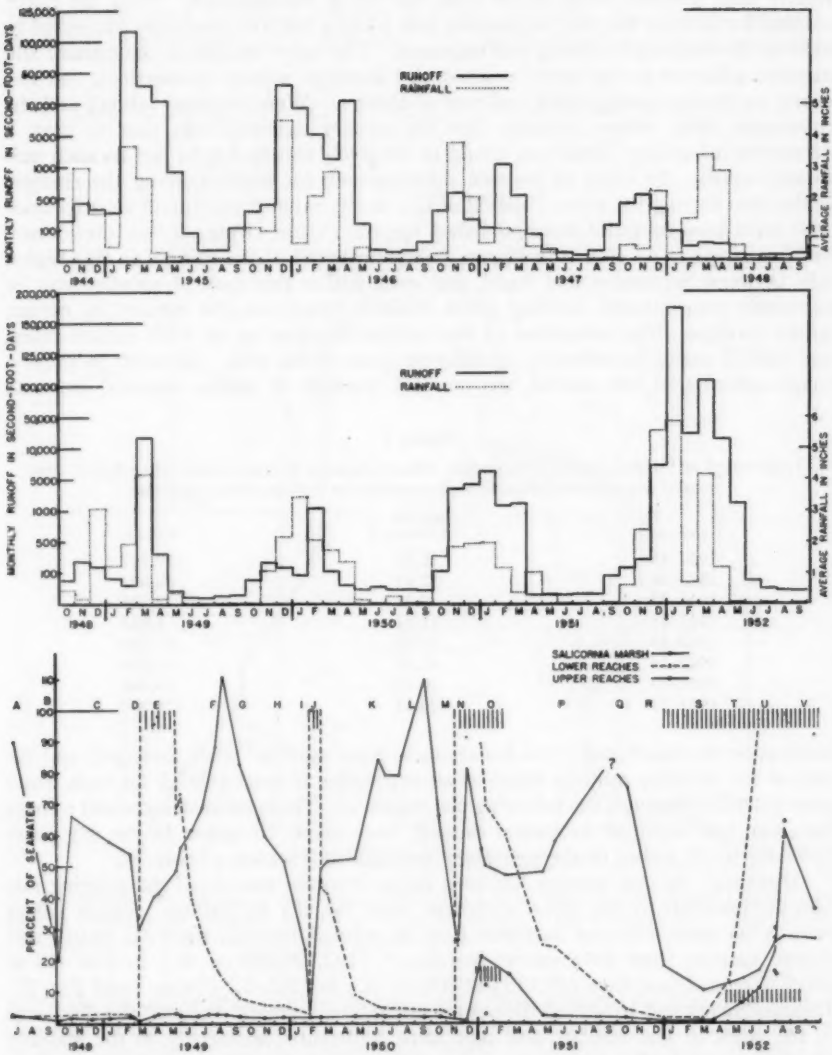

FIGURE 2. Upper and middle charts: monthly rainfall on Salinas River watershed and discharge into estuary at Spreckels, 1944–52 (note compression of runoff scale). Lower chart: salinity changes at Locations 3 (*Salicornia* marsh), 4 (upper reaches), and 1 (lower reaches), 1948–52. Letters above curves refer to points discussed in text.

Pattern of Annual Salinity Variation

It is apparent that an estuary such as that of the Salinas, in a region of variable rainfall, subject to summer diversion for irrigation and to winter floods, offers a most unstable environment, and one which can be described only in rather general terms. The observations reported below are admittedly rather superficial. The salinities are for simplicity expressed as "per cent of sea water" (taken as having a chlorinity of 19.64%, or a salinity of 35.48%). Obviously, calcium and other salts present in the distinctly "hard" Salinas River water render the river somewhat less unsuitable than its low chlorinity might suggest. This has been shown experimentally, but for purposes of giving the general pattern of variation in the brackish estuary, only chlorinity determinations have been used. Observations have usually been made at intervals of some weeks, since the Salinas lies about 100 miles from Berkeley, and were incidental to other work. Nevertheless, even scattered observations over a four-year period may contribute to an understanding of the range of variation in salinity experienced by *Neanthes lighti* in different typical habitats, and furnish a basis for appreciating the development of osmotic tolerance in a marine invader of brackish waters.

It should be noted that in the two dry winters prior to the start of this study, rainfall had been insufficient to cause the river to break through to the sea, and in the summer of 1948 the estuary was, accordingly, a quiet pond-like area of low salinity, a situation which became increasingly less stable during the following four years. Salinity determinations have been made regularly at four stations (Locations 1 to 4), and additional observations at certain others. The first three of the following stations lie in or adjacent to the lower reaches of the estuary; the last four are in the upper reaches and the head of the estuary (Fig. 1).

Location 1 represents the lower reaches of the estuary proper, below the bridges, where at times of tidal influx the salinity changes may be maximal and abrupt, but where for most of an ordinary year the water is of moderately low salinity. The bottom is of firm, somewhat muddy, fine sand, in some places underlain by clay. In these lower reaches, *N. lighti* occurs rather scatteringly and does not reach large size, a possible result of the extremely great salinity fluctuations, or of predation by wading birds at times of low tide.

Location 2 is a pool some 25 yards from the normal brink of the river, confluent with it at times of high water, but usually isolated during the summer months. *Neanthes* occurs abundantly in firm sand with an admixture of clay, black with organic matter. The salinity of the pool is variable, but is usually high, especially in summer.

Location 3 is a muddy channel winding through the *Salicornia* marsh to the south of Location 2, from which it is separated by a low roadway. This channel is isolated from the estuary except at times of flood, or at occasional high spring tides when the river mouth is open to the sea. The salinity is usually over 50% that of sea water, and may rise much higher in summer, when the channel dries up to a series of tepid pools. The bottom is of soft sandy mud, with areas of clay, quite black and foul. *N. lighti* is abundant in such areas as do not dry up entirely in summer, commonly reaching a length of 60–65 mm. preserved (Hartman, 1938, reports 45 mm. as the maximum length). The worms occupy Y-shaped burrows extending 5–8 inches into the black mud, where presumably the salinity fluctuates

far less than in the overlying water, but they are by no means insulated from this water, since the light-colored oxidized sand of the burrow walls indicates that respiratory irrigation is maintained in these passages.

The middle reaches of the estuary may be said to extend to two miles above the bridges (Fig. 1), at which point salinity variations due to tides are distinct but not extreme. At the bridges (1¾ miles from the mouth) observations made on a single day showed that salinities varied from 3.7 to 40.4% sea water over the tidal cycle. Because of the sparse population of *Neanthes* in this area, regular salinity records were not kept. Above the bridges, the estuary is narrowed and winding. Cat-tails, tulles, and willows replace the *Salicornia* and sedges of the lower reaches. By three miles from the mouth, the estuary has taken on a superficially almost fresh water aspect. Whereas the lower reaches in dry years contain *Enteromorpha, Ulva,* and encrusting green algae, the upper reaches, 4–5 miles from the mouth, contain masses of *Chara* and duckweed (*Lemna*). *Potamogeton* is abundant in both areas. Four locations in the upper reaches and the head of the estuary were studied, but at only the first of these (Location 4) were regular salinity records obtained.

Location 4 represents a widened portion of river bed with sandy bars on which *N. lighti* was extremely abundant following the dry winters of 1946–7 and 1947–8, when the area was undisturbed by flood waters and the salinity varied little. At this location, in 1948, as many as 40 adult worms could be picked from a single shovelful of sand. At times of high water, it might be difficult to obtain many individuals, while floods in the winters of 1949 and following years have swept out much of this formerly productive area. Salinities at Location 4 have remained below 3% of sea water except that as the river flow slackens in the spring occasional tidal influxes have been observed to raise the salinity temporarily to 20% of sea water or more. Conditions following the outflow of early 1952 have entirely altered this and the following upstream locations; these descriptions apply to the period of relative stability prior to 1952.

Location 5 lay about one-half mile upstream from Location 4, and represented the limit of *N. lighti,* where small numbers of reproducing worms were collected in July, 1949 (chlorinity 2.2% of sea water). This collecting spot was washed out by flood waters early in 1950.

Location 6 is about a half mile above Location 5. The intervening stretch of river was lined by cat-tails and was so narrow that in spots a skiff could be poled through only with difficulty. A current was generally perceptible in all but the driest weather, hence this region could have been considered the "head" of the estuary. Chlorinities up to 1.76% of sea water have been recorded from Location 6, which represented a sandy area just below the outfall of a drainage ditch. Although topographic maps (compiled in 1947) show the limit of permanent water in the vicinity of Location 5, I have regularly been able to take a skiff another mile upstream, there being a channel several feet deep, much narrowed by encroaching rushes and floating weeds. No *Neanthes* have ever been found at Location 6, although the substrate seems entirely suitable. However, this area is separated from Location 5 by a very muddy stretch which may have acted as a barrier to migration.

Location 7 lay at what was the limit of boat passage prior to 1952, almost a

half mile from Location 6, a little over 1.5 miles above Location 4. A slight current was usually perceptible. Chlorinities up to 1.4% of sea water have been obtained in summer. No *Neanthes* have been found, and the aspect was that of a fresh-water environment. Above this point the river channel consisted of a series of pools, separated by impenetrable beds of cat-tails.

Flood waters in the winter of 1952 swept out the masses of vegetation above Location 7, widening and deepening the channel, and permitting a boat to be taken upstream for at least another mile even at low water. Locations 5 and 7 are no longer recognizable, some bars having been swept away, others covered with many feet of sand.

The above account of conditions prevailing in the summer months at seven Locations may be amplified by presenting salinity fluctuations at three of these: Location 3, representing an area of variable but high salinity; Location 4, an area of uniformly low salinity (oligohaline); and Location 1 in the lower estuary, the area subjected to the greatest salinity changes. The data on these three representative locations have been plotted in Figure 2 (bottom) where they may be compared with each other and with rainfall and runoff at corresponding times. It is apparent that a degree of regularity in the annual pattern of salinity variation existed at each location prior to the flood year 1951–52, and that in the latter water-year conditions were profoundly altered.

At the start of field observations in 1948 (Fig. 2, A) the lower reaches (Loc. 1) were almost as fresh as the upper reaches (Loc. 4), but the isolated channel in the *Salicornia* marsh (Loc. 3) to the south of the river had a salinity of 90% of sea water, and Location 2 (not plotted) was intermediate. Early fall rains (B) in 1948 temporarily dropped the salinity at Location 3 to 20% of sea water, a loss soon largely made up by seepage and mixing. The winter rains (C) of 1948–49 produced a general lowering of salinity, culminating in early March in an inundation of the *Salicornia* marsh by nearly fresh water, followed by a break-through of the river to the sea (D). This was followed by tidal influxes (E) which caused the lower reaches (Loc. 1) to alternate between fresh and salt water, while the pool near the river (Loc. 2) became filled with sea water. With the cessation of heavy rains the flow in the river dropped to a point which permitted the reëstablishment of the bar across the mouth early in May. Although occasional high tides spilled across this bar, the lower river continued to freshen until the winter of 1950 (F, G). The pool at Location 2 remained cut off from the river throughout the summer of 1949 and retained a moderate salinity despite its proximity to the brackish river until the fall rains diluted it and eventually put it into shallow communication with the river in November 1949. The more remote pools of Location 3 became steadily concentrated by evaporation throughout the summer (F), reaching a salinity of 110% that of sea water, and then were diluted by fall rains (G).

Special interest attaches to the salinity at Location 4 during the period in 1949 when the lower estuary was subjected to tidal exchange. With the opening of the bar, the water level at Location 4 dropped until many *Neanthes* burrows were completely out of water, the surface of the sand becoming dry and sun-baked. Although the worms burrowed deeply to some 18 inches, a gradual depopulation of such exposed bars occurred; whether by lateral burrowing to lower areas or by dying off could not be determined. With each tide, but lagging by several hours,

the water level at Location 4 rose and fell about a foot. That this did not represent an influx of ocean water, but merely a backing-up and draining of fresh water, was established by repeated salinity determinations showing no difference between high and low water. In that year the bar closed in May, before the river flow became minimal. During the summer of 1949, Location 4 showed a slow rise in salinity to nearly 3% of sea water, with a gradual reduction after the fall rains (H). The winter rains of early 1950 resulted in the marshes along the lower river becoming flooded with nearly fresh water before the opening of the bar on February 8 (I). High tides coming during the succeeding period of lessened rainfall flooded the marshes with ocean water (J), raising the salinity at Location 3 to approximately 50% of sea water, but there was no indication of any salinity rise at Location 4. After closure of the bar at the beginning of March (1950), the river remained so high as to be confluent with the pool at Location 2, and both of these locations continued to freshen throughout the spring of 1950 (K). The hot late summer raised the salinity at Location 3 to 109.5% of sea water (L), but that at Location 2 remained low, without in any way seeming to harm the *Neanthes* present at either station, although reproduction seemed to be prevented by water temperatures near 30° C. on sunny days.

The fall of 1950 was marked by excessively heavy rains (M), which inundated Location 3 with nearly fresh water before the bar was opened on November 17. During December, high tides flooded the lower marshes with salt water (N), and as the river current slackened, eventually sent surges of sea water several miles upstream (O), where at Location 4 a salinity 21% that of sea water was recorded on January 6, 1951. Although by January 15 the chlorinity of the river water at this point had dropped to 2.4% of sea water, a value of 11.7% of sea water was obtained from water seeping into a hole in the sandbar from which worms were dug. The river mouth had practically closed by February 21, trapping within the lower estuary approximately 60% sea water, suggesting a considerable degree of mixing. By May, 1951, salinities at Location 4 had dropped to the usual 2.2% of sea water (P). A considerable salinity rise at Location 4, the result of spring tides at a period of slackening river flow, has been observed in two separate periods in four years, hence it very probably occurs in other years, and would seem to account for the higher "residual" chlorinities commonly encountered in water obtained from holes dug in the banks of the upper estuary. This may be an important factor in permitting *Neanthes* to survive beneath nearly fresh water.

The summer of 1951 was exceptionally cool and cloudy (Q). Salinity records were unavoidably interrupted, but the river remained high, confluent with the pool at Location 2, while the water in channels back from the river (Location 3) apparently did not become as concentrated by evaporation as in typical summers. Heavy December rains (R) flooded the lower marshes, and the river broke through the bar on December 30, 1951. A visit to Location 4 on January 1, 1952 showed a swirling muddy torrent pouring to the sea. Such sand bars as could be approached were found to be shifting masses of quicksand, indicating a heavy loss of *Neanthes* from this area. The great fresh water discharge during early 1952 signaled the end of the previous ecological distinctness of Locations 3 and 4. The flow into the lower reaches was high enough to inundate the *Salicornia* marshes with water of low salinity (S)—at least there is no evidence of the flooding of the marsh with sea water from a high tide at a time of slackening river flow as had

been the case in previous springs, although the Location 2 pool did receive tidal influxes. The result was that Location 3 remained at a very low salinity, and did not in the entire summer of 1952 attain a salinity in excess of 27% sea water (S, T). Concommitantly *Neanthes* virtually disappeared from Location 3; only a few very small worms were found by careful search in July. However, Location 2, which had received influxes of sea water, retained the usual pattern of high spring and summer salinity (reaching 135% of sea water on July 30), and continued to support a normal population of *Neanthes*. It is difficult to avoid the impression that the worms of Location 3 suffered heavy mortality as a result of several months of salinities below 20% of sea water.

In the case of Location 4, the destruction of the worm population was nearly complete (only a single worm rewarded several hours of intensive search on July 31, 1952). But here the loss may be more attributable to the extensive erosion, widening, and deepening of the channel at Location 4 by several months of high stream flow than to adverse effects of salinity change. With the slackening of river flow in the spring of 1952 (T), and continuing through the summer (U), tidal effects became regular in the opened river channel. The mouth of the river remained open throughout the summer of 1952, apparently as a result of tidal scouring, for the flow was not much in excess of normal. Possibly wave action in the summer months is insufficient to throw up the barrier bar if a high discharge by the river in the spring has prevented formation of the bar at the usual time. As a result, the salinity at Location 4 rose and fell more or less regularly, a range of 15 to 30% sea water being noted in the course of a single moderate tide; probably the larger tides had considerably greater effects.

The fact that some worms evidently survived the summer of 1952 in both Locations 3 and 4, and survived in normal abundance at Location 2, suggests that in future years of low rainfall we may again see conditions stabilized and new populations of *Neanthes* developed as before. The evolutionary interest pertaining to such massive depopulations followed by repopulation from a few survivors is obvious, and a re-study of physiological adaptions in the new populations, if such arise, should be made to extend the preliminary studies carried out upon the past populations.

Faunal Associates of Neanthes lighti

In the summers of 1949 and 1950, before the stabilized "dry-year" pattern of the Salinas estuary had been upset, collections of the fauna at the several stations were made. Although conditions have in some instances been altered drastically, the following notes give a picture of the estuarine fauna in a condition as near equilibrium as is likely to be found. Since the range of *N. lighti* shows such marked variation in salinity, and includes areas with a distinctly fresh water aspect, a tabulation of the animals associated at a given location may be of value in estimating the relative extent of the penetration of this worm into "fresh" water. The statement of Hartman (1938) that *N. lighti* may occur in freshwater pools, as judged by the associated fauna of insects, etc., should be checked by a study of actual salinities. As is shown below, the faunal associates of *N. lighti* in the Salinas River include both fresh water and brackish water types.

Location 1: Most common were the isopod *Neosphaeroma oregonensis,* and the

amphipods *Corophium spinicorne* and *Anisogammarus confervicolus*. The chief planktonic crustacean was *Neomysis mercedi*, while insects were represented by corixids and dipteran larvae. The openness of the area and the abundance of waders and sea-birds may contribute to a general paucity of fauna.

Locations 2 and 3: These contain in general the same fauna as Location 1 except the *Neomysis* is absent from both and *Corophium spinicorne* scarce at Location 3, especially at periods of high salinity. The small snail *Amnicola* occurs in large numbers on *Potamogeton* and other plants at Location 2.

Location 4: At this and the following points, collections were made from among cat-tails as well as from the adjoining sand, so that the habitats examined in the upper reaches are comparable. The fauna included the following typically fresh-water forms: *Physa, Chironomus, Plumatella, Hyalella azteca,* dragon-fly larvae, several dipteran larvae, bellostomid and corixid bugs, and beetle larvae. In addition, the following forms of marine affinities were well represented: *Corophium spinicorne, Anisogammarus confervicolus, Neosphaeroma oregonensis,* and *Neomysis mercedi.* Careful search revealed no planarians but a few leeches (*Placobdella fusca*).

Location 5: A small coarse sand beach contained small numbers of breeding *Neanthes* in July, 1949 (salinity 2.2% sea water). Collections in this area included essentially all the fresh water types encountered at Location 4, plus the snail *Gyraulus,* ephemerid and damsel-fly larvae and a few small planarians. *Corophium spinicorne* and *Neosphaeroma* were present but scarce. *Anisogammarus* was absent.

Location 6: Below the point where fresh water drainage ditch empties into the river, a sand bar suitable for *Neanthes* was examined, but found to harbor only chironomids and *Corophium stimpsoni,* the latter breeding and present in large numbers. Since the bottom appeared suitable and the chlorinity possibly adequate (1.75% of sea water), the absence of *Neanthes* may have been due to the long stretch of river above Location 5 which lacked suitable bottom for these worms. The fauna also differed from that at Location 5 in the absence of *Neosphaeroma* and of *Corophium spinicorne.* The replacement of the latter by *C. stimpsoni* is of especial interest. Shoemaker (1949) states that *C. spinicorne* is the only *Corophium* recorded from the fresh waters of America, but the presence of *C. stimpsoni* in the freshest part of the Salinas estuary suggests that the later species is actually more tolerant of fresh water than is *C. spinicorne.*

Location 7 was at the limit of boat passage, approximately ½ mile from Location 6 (2 miles from Location 4). The fresh water fauna previously encountered were here in abundance, including numerous leeches and very abundant large planarians (*Dugesia* sp.). The only representative of the "marine" fauna was *Neomysis,* present in great numbers. Chlorinity was 1.4% that of sea water. The bottom was muddy and no *Corophium* were taken.

This cursory account suffices to show that the range of *Neanthes lighti* coincides in a general way with those of *Neosphaeroma oregonensis, Anisogammarus confervicolus,* and *Corophium spinicorne,* all of which are characteristic brackish water species. Conversely, it appears that *N. lighti* occupies a region which overlaps only slightly the ranges of such fresh water forms as planarians or leeches, although numerous fresh water insects, as well as the snail *Physa,* extend into the

less saline portions of the range of *Neanthes*. It would appear on the basis of these scanty collections that *Corophium stimpsoni* may be restricted to waters less saline than those tolerated by *Neanthes lighti*, but final judgment on this point should await a detailed study of the distribution of the several species of *Corophium* within brackish waters. Certain forms, such as *Neomysis mercedi* and the groups of the corixids and chironomids, have a much wider salinity tolerance than *N. lighti*, and are not so useful as indicators in this situation.

Discussion

While Figure 2 upon first inspection seems to reveal a situation little short of chaotic, the observations over the past several years permit certain generalizations concerning the conditions of life in a small California estuary. There are, most strikingly, wide and irregular salinity variations in the lower reaches, these being most marked in the lower river channel itself, where long periods of quiet water of low salinity are interspersed with weeks or months of tidal action which may result in a daily salinity shift from 3% to 100% of sea water, as well as exposure to air and to predators. It is perhaps significant that in this area of most extreme change and exposure hazard, *Neanthes lighti* is least abundant.

These worms have been more consistently abundant, larger, and more vigorous in two general situations, differing in opposite senses from the lower estuary proper. The following remarks apply to conditions prior to the upset of 1951–52, which is considered to have been a decidedly abnormal season. At Location 3 there has usually been a salinity range of 30–40% to above 100% that of sea water. Owing to the tendency of fresh water to layer out above salt water, and for substrata of high salinity to be little affected by submergence beneath fresh water for relatively short periods (Reid, 1930, 1932), it does not seem likely that worms in their burrows in the muddy channel at Location 3 are ever abruptly exposed to salinities as low as are measured in the river itself at times of flood, or at most for very limited periods. Worms from this area can be adapted to chlorinities equivalent to 1% of sea water; hence salinity variations at Location 3 are not extremely severe. Temperatures of 30° C. in summer seem not to injure the adult worms, but result in failure of fertilization and/or development of embryos (Smith, 1950).

In marked contrast has been the prevailing low salinity at Location 4 in normal years. Over most of the period of observation, chlorinities have varied from about 3% of sea water down to as low as 0.25% at times of flood. Worms in their burrows perhaps receive protection against the lower salinities of the flowing water above them as a result of "residual" salinity in the sand of the banks and bottom. It has commonly been noted that worms collected at times of flood become much swollen if retained in river water, but survive better if kept in the sand from which they were dug. Such worms tend to form distinct "jackets" of mucus and sand, which may offer some protection. It has also been found best to keep worms collected from brackish water at room temperature, since refrigeration apparently retards active osmoregulation to such a degree that swelling and death may occur. Mixing from the lower reaches and occasional tidal influxes of sea water probably serve to maintain the slight salinity required for survival in such nearly freshwater areas in normal years.

It is apparent that *N. lighti* is a brackish-water type which has not completed the

transition to a strictly fresh water habitat. From a study of conditions in the Salinas River it is not possible to say whether this transition could be made. From the fact that the range of *N. lighti* seems to coincide with that of such forms as *Corophium spinicorne* and *Neosphaeroma oregonensis,* but does not extend so far upstream as that of *Corophium stimpsoni* or *Neomysis mercedi,* we may judge that it is actually limited in its osmoregulatory abilities. Furthermore, the dropping out of such typically fresh water forms as odonata larvae, planarians, and leeches as the fresh water fauna approaches the upper limit of *N. lighti* suggests that the salinities required by *N. lighti* are not inappreciable, inasmuch as they may limit the seaward extension of these fresh water animals.

Physical barriers to the upstream spread of *N. lighti* appear serious, but are difficult to evaluate precisely. Recurrent rushes of flood water have been observed to sweep away well-populated sand bars, or to cover productive areas with thick layers of barren sand. Thus the bar at Location 5, which in 1949 marked the upstream limit of *N. lighti,* was completely washed away in February, 1950, and the area has not been repopulated. Another factor which may have limited the spread upstream beyond Location 5 is the fact that the stream above this point became thickly bordered by cat-tails growing in very soft black mud. *N. lighti* seems to require a sandy substrate for its burrows, and has never been taken in the sort of mud found for the half mile above Location 5. There is usually a perceptible current in this area, even in summer in dry years, which may have further discouraged upstream migration of young worms.

The nereid population of the upper reaches has been in several senses isolated from that of the *Salicornia* marshes: First, by the fact that the normal range of salinities at Location 4 does not (except for infrequent and brief periods) overlap the range of salinities at Location 3. Second, by the presence of intervening stretches of river physically poorly suited for large populations of *Neanthes.* Third, by the fact that *N. lighti* appears to consist of self-fertilizing hermaphroditic viviparous individuals, which do not produce widely-dispersing planktonic larvae. The problem of whether or not these populations constitute actual physiological races requires more detailed study. Preliminary studies have been made to ascertain this point, but since the studies cannot be confirmed until conditions in the estuary have become stabilized and new populations developed, only an abstract of these studies need be included here. In this work, the respiration of worms from Location 4 was compared with that of worms from Location 3. The method used was to adapt worms from each area gradually to a series of different salinities (1, 5, 10, 50, 100, and over 100% of sea water), and to maintain them (with feeding) at these salinities for three or more weeks to ensure full adaptation. It was considered that the worms became nearly as fully adapted to the test salinities as their physiological constitutions would permit. It was found that worms from Location 3, adapted to salinities 5% or less of sea water showed a higher respiration on the average than did worms from Location 4 adapted to identical media. Since a number of variables, including size, had to be taken into account, the results, although apparently significant, are considered indicative of, rather than conclusive evidence for, the existence of physiologically distinct races of *N. lighti* in different parts of the Salinas estuary. The drastic reduction of the populations in question following the hydrographic upset of 1951–52 further suggests that these populations were physiologically specialized or distinct. Because the upset of

stable conditions will delay completion of these studies for some time, until the estuary stabilizes, the work will not be reported in detail until it can be confirmed.

Summary

1. Salinity and salinity changes have been studied over the range of the polychaete *Neanthes lighti* Hartman in the estuary of the Salinas River, California, during the years 1948–1952.

2. Not only are mean salinities different in various parts of the range of *N. lighti*, but each locality has a characteristic annual pattern of salinity variation depending upon fresh water discharge into the estuary.

3. The pattern of salinity variation is traced through years of low and moderate rainfall, and its upset during the flood season of 1951–52 is reported.

4. Worms inhabiting marshy areas near the river mouth are exposed to variable but high salinities (40 to over 100% of sea water); those in the upper reaches endure uniformly oligohaline conditions (2–3% of sea water) for most of the year. Either of these areas is more favorable than the lower reaches of the estuary that receive full tidal exchanges for part of each year.

5. Over its range, *N. lighti* is associated with such typically brackish water species as *Neosphaeroma oregonensis* (Dana), *Anisogammarus confervicolus* (Stimpson), and *Corophium spinicorne* Stimpson. Its range only slightly overlaps the ranges of such fresh water forms as *Dugesia* sp., leeches, or odonata larvae. *Corophium stimpsoni* Shoemaker is found in water fresher than that inhabited by *N. lighti* or the crustaceans associated with it.

6. The populations of *N. lighti* of the upper reaches are ecologically and reproductively isolated from those of the more saline marsh channels, and some evidence for the existence of physiological distinctness between these populations has been found.

LITERATURE CITED

Day, J. H., 1951. The ecology of South African estuaries. Part I. A review of estuarine conditions in general. *Trans. Roy. Soc. South Africa,* **33**: 53–91.

Hartman, O., 1938. Brackish and fresh-water Nereidae from the northeast Pacific, with the description of a new species from central California. *Univ. Calif. Publ. Zool.,* **43**: 79–82.

MacGinitie, G. E., 1935. Ecological aspects of a California marine estuary. *Amer. Midl. Nat.,* **16**: 629–765.

Reid, D. M., 1930. Salinity interchange between seawater in sand and overflowing freshwater at low tide. *J. Mar. Biol. Assoc.,* **16**: 609–614.

Reid, D. M., 1932. Salinity interchange between salt water in sand and overflowing freshwater at low tide. *J. Mar. Biol. Assoc.,* **18**: 299–306.

Scott, K. M. F., A. D. Harrison and W. Macnae, 1952. The ecology of South African Estuaries. Part II. The Klein River Estuary, Hermanus, Cape. *Trans. Roy. Soc. South Africa,* **33**: 283–331, pl. 25–26.

Shoemaker, C. R., 1949. The amphipod genus *Corophium* on the west coast of America. *J. Wash. Acad. Sci.,* **39**: 66–82.

Smith, R. I., 1950. Embryonic development in the viviparous nereid polychaete, *Neanthes lighti* Hartman. *J. Morph.,* **87**: 417–465.

U. S. Department of Commerce Weather Bureau, "Climatological Data, California Section." 1944–1952.

U. S. Department of the Interior, Geological Survey Water Supply Papers, "Surface Water Supply of the United States," Part 11, Pacific Slope Basins in California, 1944–1952.

QUANTITATIVE STUDY ON THE REGIONAL DISTRIBUTION OF PENTOSE NUCLEIC ACID IN THE GASTRULA AND NEURULA OF TRITURUS

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The aim of the present study is to estimate chemically the amount of pentose nucleic acid in the different prospective regions of the ectoderm and mesoderm in the early gastrula and the early neurula of Amphibia, and to obtain quantitative information about its spatial distribution and its change during gastrulation. The eggs of *Triturus pyrrhogaster,* which were found to satisfy special demands of our study, were used exclusively. In this material the prospective significance and the morphogenetic movement of the different regions have been worked out in detail (Nakamura, 1942) and operative separation of these regions can be successfully carried out with techniques developed in this laboratory.

Material and Method

The estimation was carried out on the following developmental stages: (1) gastrula with beginning blastopore, corresponding approximately to stage 11 of Okada and Ichikawa's table of *Triturus pyrrhogaster* (1947) and to stage 10 of Harrison's table of *Amblystoma punctatum;* (2) neurula, shortly after the formation of the neural folds, corresponding to stage 16 of Okada and Ichikawa's table and to stage 14 of Harrison's table.

Following the removal of the capsule and vitelline membrane the embryos were operated on in Holtfreter's solution on a Schotté ring using the glass needle and glass spherule. For a better separation of the germ layers the operation dish was kept cold with ice. During the operation utmost care was taken to obtain isolates of pure prospective significance, free from contamination by cells from the adjoining regions and underlying layer.

In the gastrula the following regions were isolated for estimation:

(a) The prospective ectoderm (A, Fig. 1a), consisting of the prospective neural plate (the cross-hatched part of A) and the prospective epidermis (the plain part of A). In a special series the prospective neural plate and the prospective epidermis were estimated separately.

(b) The dorsal marginal zone (B, Fig. 1a), containing the prospective notochord and the dorsal half of the prospective somites. The prospective pre-

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chordal material, which is continuous with the yolk-rich endoderm of the pharyngeal wall, without a clear boundary, was not included.

(c) The lateral marginal zone (C, Fig. 1a), containing the ventral part of the prospective somites and the prospective lateral plate.

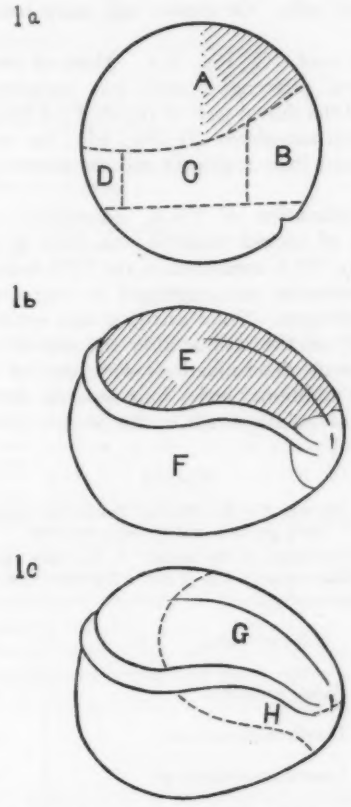

FIGURE 1. Schemes for isolation of samples for PNA estimation. 1a: Gastrula; A: prospective ectoderm (the cross-hatched half is the prospective neural plate, the plain half the prospective epidermis); B: dorsal marginal zone; C: lateral marginal zone; D: ventral marginal zone. 1b: Neurula; E: neural plate (cross-hatched area); F: epidermis (neural fold, and area surrounding the blastopore are omitted). 1c: Neurula; G: dorsal mesoderm; H: lateral and ventral mesoderm.

(d) The ventral marginal zone (D, Fig. 1a), containing the prospective blood island and adjoining ventral mesoderm.

From the neurula the following regions were estimated:

(a) The neural plate (E, Fig. 1b). The neural folds were not included. Also the most caudal one-fifth of the neural plate which contained the prospective

somites (Nakamura, 1942) was omitted. The cells of the archenteric roof, which were sticking to the internal surface of the neural plate, were removed very carefully with the finest glass spherules.

(b) The epidermis (F, Fig. 1b). Only very careful operation gave pure epidermis without attached mesoderm cells, which were more difficult to remove than the archenteric roof cells. Of course only pure isolates were used for the estimation.

(c) The archenteric roof (G, Fig. 1c). Most of the prechordal plate was omitted. No endodermal pharyngeal wall was included. This region corresponded to region B and the dorsal part of region C of the gastrula.

(d) The latero-ventral mesoderm (H, Fig. 1d); the mesoderm underlying the epidermis. This is derived from region D and the ventral part of region C of the gastrula.

For the chemical estimation of PNA, Schneider's technique (1945) was adopted. The intensity of orcinol reaction was read by a Pulfrich spectrophotometer. Interference by DNA contained in the "PNA-fraction" was found to be negligible. PNA concentration was expressed in micrograms of phosphorus per milligram of the total nitrogen. Total nitrogen was estimated by the method of Levy and Palmer (1940) on the residue after the nucleic acid extraction. In the principal series six separate determinations were carried out for each region of both stages, while in the additional series four separate determinations were made. Thirty-five to 45 pieces of isolates made up the sample for one estimation.

TABLE I

PNA P_γ per mg. N of the different prospective regions of the early gastrula and the early neurula

$\overline{X}$: mean; $S\bar{x}$: standard deviation of the mean; **: F_0-value significant at 1% level; *: F_0-value significant at 5% level but not so at 1% level; °: the same value not significant on 5% level.

Stage	Prospective regions	PNA P_γ/mg. N $\overline{X} \pm S\bar{x}$	Ratio of variances F_0
Gastrula	(A) Prosp. ectoderm	9.6±0.60	28.93**
	(B) Dorsal marginal zone	6.0±0.30	11.42*
	(C) Lateral marginal zone	4.8±0.11	0.25°
	(D) Ventral marginal zone	4.6±0.33	
	(A′) Prosp. neural plate	11.5±0.28	10.98*
	(A″) Prosp. epidermis	9.7±0.47	
Neurula	(E) Neural plate	13.8±0.68	7.88*
	(F) Epidermis	11.1±0.66	11.83**
	(G) Dorsal mesoderm	7.7±0.73	0.01°
	(H) Latero-ventral mesoderm	7.6±0.74	

TABLE II

Comparison of PNA Pγ/mg. N of prospective regions of the gastrula and neurula. Compare with Table I

Prospective regions	Ratio of variances F_0
Prospective neural plate of gastrula / Neural plate of neurula	6.9*
Prospective epidermis of gastrula / Epidermis of neurula	2.67°
Prospective ectoderm of gastrula / Neural plate of neurula	20.67**
Prospective ectoderm of gastrula / Epidermis of neurula	2.83°

RESULTS

The principal data obtained are summarized in Table I.

Gastrula. The prospective ectoderm (A) showed the highest value, while the dorsal marginal zone (B) gave a value significantly less than the former. The dorsal marginal zone, the organizer region, in its turn showed PNA content higher than the lateral and ventral sections of the marginal zone. This difference was significant at the 5% level, but hardly so at the 1% level. The latter two sections failed to give any significant difference.

In the additional series PNA content of the prospective neural plate and the prospective epidermis was estimated (Table II). The results showed a difference significant at the 5% level in favor of the prospective neural plate.

Neurula. The neural plate (E) showed the highest value, significantly higher than the dorsal and latero-ventral mesoderm of the same stage (G and H). The difference between the neural plate (E) and the epidermis (F) was found to be significant at the 5% level, but not at the 1% level. No significant difference could be detected between the dorsal and latero-ventral mesoderm.

DISCUSSION

The present data show clearly that in both stages examined the ectoderm contains more PNA than the mesoderm, if the amount is calculated on the over-all nitrogen base. The organizer region, *i.e.*, the dorsal marginal zone of the gastrula and the archenteric roof of the neurula, contain significantly less PNA than is contained in the ectoderm in both stages. Within the ectoderm the neural plate and its prospective region seem to possess more PNA than the epidermis and its prospective region, although the difference obtained is significant only at the 5% level. Thus, our data make it very probable that the apex of the gradient of the substance throughout both germ layers is present in the material of the neural system in the stages studied. Comparing the data on ectodermal regions of both stages it might be pointed out that during gastrulation an accumulation significant at the 5% level can be detected for the neural plate region, but not for the epidermis region.

As to the marginal zone our data show a difference significant at the 5% level

between its dorsal section and the rest of the zone in the gastrula. However, in the neurula no significant difference can be shown between the dorsal and latero-ventral mesoderm.

If we compare the data obtained from different regions with the inductive effect of these regions on the presumptive ectoderm, it is clear that the simple over-all PNA concentration of any germ region as such does not decide the inductive capacity of that region. The epidermis of the neurula is non-inductive and the ectoderm of the gastrula is very weakly if at all inductive, but they contain more PNA than the organizer itself. In the early neurula the archenteric roof has stronger inductive effect but not the latero-ventral mesoderm. However, they all have about the same amount of PNA. Furthermore this amount is decisively less than that of the non-inductive epidermis.

As to the spatial distribution of PNA within the different prospective regions of the amphibian embryo, until now only data based on the observation of the histochemical section have been available. The general features of the gradient as demonstrated by histochemical techniques by Brachet (1942, 1947), such as the animal-vegetal and dorso-ventral gradient, are mostly in accord with our present data. However, no indication was obtained in the present study of a high concentration of PNA in the organizer. The present author studied the PNAase-sensitive basophilia in embryos of *Rhacophorus schlegelii* (anura, pigmentless eggs; Takata, 1950), *Triturus pyrrhogaster* and *Megalobatrachus japonicus* (urodele, pigmentless eggs; Takata, unpublished results). During gastrulation the apex of the gradient was found always in the prospective neural plate. The dorsal marginal zone was stronger in intensity than the lateral and ventral marginal zone, but decisively weaker than the ectodermal regions. These observations are in good accord with the present data and there seems to be no ground to support the idea that the organizer represents the apex of the PNA gradient, if its value is calculated on the basis of over-all nitrogen. However, it is still an undecided question whether the PNA concentration of the active protoplasm (*i.e.*, PNA calculated on the basis of the non-yolk nitrogen) has its highest value in the organizer region.

Insofar as data on total nitrogen basis are concerned we did not find any indication for decrease of PNA in the invaginating archenteric roof, as suggested by Brachet from his histochemical sections.

Comparing the data on two stages, a general increase of PNA content in both layers during gastrulation is evident. This rise seems to be in accord with the data on the total PNA amount of amphibian embryos during the early development, where more or less clear increase of the value beginning from gastrulation was noticed (Brachet, 1941; Steinert, 1951).

Summary

1. PNA content of prospective regions of the ectoderm and mesoderm of the early gastrula and neurula of *Triturus pyrrhogaster* was estimated by the technique of Schneider (1945) and expressed in microgram phosphorus per mg. nitrogen.

2. At both developmental stages PNA content was higher in the ectodermal regions than in the mesodermal regions. During gastrulation, an increase in PNA amount was noticed for most of the regions studied.

3. In the gastrula the highest amount of PNA was found in the ectoderm (prospective neural plate) but not in the organizer region. In the marginal zone the dorsal region showed an amount higher than that of the lateral and ventral marginal zones.

4. In the neurula the neural plate showed the highest value. The main part of the archenteric roof contained less PNA than the epidermis. No significant difference could be detected between the values of the archenteric roof and the rest of the mesoderm.

LITERATURE CITED

BRACHET, J., 1941. La detection histochimique et le microdosage des pentose nucléiques. *Enzymol.*, **10**: 87–96.

BRACHET, J., 1942. La localisation des acides pentose nucléiques dans les tissues animaux et les oeufs d'Amphibiens en voie de développement. *Arch. de Biol.*, **53**: 207–257.

BRACHET, J., 1947. The metabolism of nucleic acids during embryonic development. *Cold Spring Harbor Symp.*, **12**: 18–27.

LEVY, M., AND A. H. PALMER, 1940. Iodometric estimation of small quantity of nitrogen without distillation. *J. Biol. Chem.*, **136**: 57–60.

NAKAMURA, O., 1942. Die Entwicklung der hinteren Körperhälfte bei Urodelen. *Annot. Zool. Japon.*, **21**: 170–235.

OKADA, Y. K., AND M. ICHIKAWA, 1947. Tables of developmental stages of *Triturus pyrrhogaster*. *Annual Rep. Exp. Morphol.* (*Tokyo*), **3**: 1–6.

SCHNEIDER, W. C., 1945. Phosphorus compounds in animal tissues. I. Extraction and estimation of desoxypentose nucleic acid and of pentose nucleic acid. *J. Biol. Chem.*, **161**: 293–303.

STEINERT, M., 1951. La synthèse de l'acide ribonucléique au cours du développement embryonnaire des batraciens. *Bull. Soc. Chem. Biol.*, **33**: 549–553.

TAKATA, K., 1950. On the distribution of RNA within the amphibian embryo. (In Japanese.) *Zool. Mag.* (*Tokyo*), **59**: 28.

ABSTRACTS OF PAPERS PRESENTED AT THE MARINE BIOLOGICAL LABORATORY

1953

ABSTRACTS OF SEMINAR PAPERS

JUNE 30, 1953

The structure of ribonuclease.[1] DOROTHY WRINCH.

Preliminary X-ray studies of crystalline proteins have been made by many investigators from which only broad inferences regarding symmetry and volume of the protein entities can be made. However, for a few crystalline proteins, intensity data have been obtained. In such cases information regarding the structures of the proteins can be obtained if, and only if, it proves possible to interpret the intensity map or its equivalent, the vector map. The fundamental question relating to a particular protein entity for which it is feasible to look for answers in intensity or vector maps at this time concerns *the type of atomic pattern* adopted by the sets of interlocked N-C_α-C monomers which constitute the skeletons of the molecules of the entity in question.

A more detailed study of the vector maps of ribonuclease given by Carlisle and Scouloudi (1951, 1953) now confirms the suggestion (Wrinch, 1952) that this protein does not have the classical type of structure comprising a bundle of polypeptide chains and that, on the contrary, the data lend themselves to interpretation in terms of skeletons which are genuinely three-dimensional in character. The nature of the vector maps suggests that these structures are indeed globulite, *i.e.*, more or less equi-dimensional in shape, that they are cage-like and that they have low-density interiors. From Perutz's X-ray data on horse hemoglobin (1949), corresponding conclusions were reached (Wrinch, 1952, 1953). Comparing the results we encounter the striking fact that, for these two different proteins, the X-ray data may be interpreted in terms of skeletons which are not only of the same type but are even of about the same dimensions, having diameters of the order of, say, 10–15 Å.

An Approach to the problem of DNA constancy.[2] W. S. VINCENT.

The DNA (desoxyribonucleic acid) content of individual nuclei is usually determined by microphotometry of the Feulgen stain. Such measurements usually give, within one ploidy class, a normal distribution curve with a variation of about 30% of the mean. If one measures the dye bound in each of the two nuclei of a binucleate cell one might expect to sensibly control variations due to spatial effects of fixation and staining. The variation between two nuclei within a single cytoplasm should be less than that found between two randomly located nuclei if errors due to staining and fixation procedures are present. Variation due to instrument errors can be determined by repeated measurements on the same nucleus.

Tissue from the median lobe of a mouse liver fixed in alcohol-acetic, formalin-sucrose and Zenker-formol was hydrolyzed and stained for maximum color development in a standardized Feulgen procedure. Two measurements were made on each nucleus of fifteen binucleate cells from each fixative. Analysis of variance and calculation of the distribution of the variation gave the following results: Variation (1) due to measurement, 2%; (2) due to fixation, 3%; (3) between nuclei in the same cell, 47%; (4) between nuclei in different cells, 48%.

[1] This work is supported by the ONR under contract with Smith College.
[2] Supported by AEC project 1187, administered by Dr. John W. Gowen.

Two possible explanations for the finding that nuclei in the same cell have the same variation as nuclei in different cells are given: (1) The normal distribution curve of DNA content found in microphotometric measurements on a cell population is a true picture of the content of this substance in the individual nuclei. This explanation implies that the hypothesis of DNA constancy is true only in a statistical sense for a given population of nuclei. (2) An alternate explanation is that the Feulgen procedure is subject to random and as yet undefined variations in combining with DNA. Such an explanation negates the value of the Feulgen reagent in accurately determining DNA content of single cells.

Chemical and electron microscopic observations on the isolated mitotic apparatus. DANIEL MAZIA.

The method of isolating the mitotic apparatus from dividing sea urchin eggs that was described earlier by Mazia and Dan involved three steps: (1) preservation of the intact apparatus in subzero 30 per cent ethanol, (2) stabilization by means of an oxidizing agent, and (3) dispersal of the cytoplasm surrounding the mitotic apparatus by means of the detergent Duponol. Step (2) undoubtedly causes drastic alteration of the proteins and Duponol also is known to denature many proteins. The method has now been modified by the elimination of step (2), which is possible if a much gentler detergent than Duponol—digitonin—is employed to disperse the cytoplasm.

The newer and gentler method of isolation, applied to the eggs of *Strongylocentrotus purpuratus*, yields intact mitotic apparatus with the chromosomes as well as the spindles, asters, and centers preserved in a seemingly normal condition. The chromosomes retain their DNA, and the protein isolated from the apparatus also contains about 2% RNA, which had not been found in the earlier preparations. The mitotic apparatus isolated by the digitonin method is soluble in 1% sodium thiogylcollate at pH 11.5. At pH 11.0 the thioglycollate dissolves the spindle and the radiating fibers of the aster, leaving behind the centrosphere and that part of the aster immediately surrounding the centrosphere. It is thus possible to isolate this part of the mitotic apparatus.

Electron microscopic observation reveals the mitotic apparatus to be a gel in which the fibers represent regions of higher orientation.

JULY 7, 1953

Neuronal derivatives of the neural crest in the chick. WARNER S. HAMMOND.

Experiments in which the neural tube or the neural crest of the chick embryo were removed indicate that the appearance of sympathetic ganglia of the neck and trunk is dependent upon the presence of cells from the neural crest of the region. In addition, hindbrain crest is found to be the source of cells for the intrinsic ganglia of the heart, lungs and digestive tract.

Independent differentiation of preganglionic and postganglionic neurons of the sympathetic system is also indicated by the experiments.

Neurons of the dorsal root ganglia are absent following extirpation of the neural crest and/or neural tube of chick embryos up to 28 somites in age. Failure of these neurons to differentiate after neural tube removal may be due to loss of their connection to the central nervous system.

The migration of morphologically undifferentiated cells from the neural crest occurs in a longitudinal direction as well as a dorso-ventral one. The factors responsible for migration, localization and subsequent differentiation of crest cells into neurones remain obscure. Kappers' theory of neurobiotaxis does not appear to be applicable in this situation.

The regression-replacement cycle of hydranths in Obelia and Campanularia. SEARS CROWELL.

Continuous observations of rapidly growing, healthy colonies of *Campanularia flexuosa* show that individual hydranths are short-lived: after a few days a hydranth loses its form; its substance passes into the coenosarc of the colony; the hydrotheca drops off; and in a day

or two a new hydranth, with a short pedicel, develops at the tip of the old pedicel. On the average, feeding hydranths persist for about 4 days at 21–22° C. and for about 7 days at 17° C. The process is orderly in that older hydranths undergo regression before younger ones. A five-day starvation period accelerated the regression cycle but did not alter the orderliness of the sequence.

Original data sheets of F. S. Hammett show that older hydranths undergo regression much more frequently than younger ones when colonies are warmed for 24 hours. Ordinarily commercial slides of Obelia not only show stages of regression and replacement but also indicate the orderliness of regression. Older parts of the stem have pedicels whose perisarcal annulations show several distinct thicknesses. These are the record left by several cycles of regression and replacement.

Brief observation of a few other hydroids suggests that a natural cycle of regression and replacement is common in calyptoblasts. Although hydranths of gymnoblasts undergo regression this is not known to occur under favorable conditions. This may be related to the fact that once a calyptoblastic hydranth is developed it does not grow while a gymnoblastic hydranth grows extensively after becoming a small feeding hydranth.

The naturally occurring cycle of regression and replacement may have some advantage in that attaching organisms are removed by shedding of the hydrotheca. Probably the principal significance lies in the fact that a colony is able to avoid senescence by replacing old with new, and is able, by resorption of the substance of the old, to do this without loss to the colony as a whole.

JULY 14, 1953

Potassium and sodium movements in rabbit polymorphonuclear leukocytes. H. G. HEMPLING.

Studies were carried out on the electrolyte metabolism of rabbit polymorphonuclear leukocytes obtained by intraperitoneal injection. Potassium content was found to be 105.1 ± 6.1 meq/kg. cell water, sodium 67.5 ± 11.1 meq/kg. cell water, and the water content 79.0 ± 1.14 per cent. Simple cytolysis of the cells in distilled water removed all analyzable K and Na, and ashing was found to be unnecessary. Cells in exudate fluid with an average K concentration of 2.0 meq/liter were found to lose K gradually over a 24-hour period at 37° C., replacing the K with a reciprocal amount of Na. Usually 50% of the cell K was lost during this time period. The addition of glucose was found to slow the rate of K loss, regardless if the glucose were added initially or at intermittent intervals. Studies on glucose utilization and hydrogen ion production were carried out in conjunction with electrolyte analyses. Protection by glucose against K loss could not be attributed to simple hydrogen ion exchange for extracellular K. Maintaining cell suspensions at 2° C. produced an enhanced loss of K as compared to losses at 37° C., described by the equation of a first order reaction. Per cent of cell K lost per hour was 5.76. An exact reciprocal of Na was gained. Systems allowed to lose K at 2° C. when placed at 36° C. took up K and extruded Na in a reciprocal fashion against concentration gradients. Approximately 100 meqs K/kg. cell water were transported in two hours from an environment of 3 meqs/liter.

Clotting of fibrinogen as a problem of protein chemistry. L. LORAND.

Studies on fibrinogen have established the basic principles involved in the clotting phenomenon. The alteration that fibrinogen undergoes prior to aggregation under the influence of thrombin has been characterized by a species specific change in the N-terminal residues of the protein (from glutamic acid to glycine in bovine, and alanine to glycine in human material) and the liberation of non-protein nitrogen (ca. 3%) in the form of fibrino-peptide. Fibrinopeptide is an acidic unit of the fibrinogen molecule and represents, at physiological pH, highly charged negative centers. The removal of these centers by thrombin would eliminate some repelling charges from fibrinogen, and make isoelectric sites on the protein that could serve

as contact points for neighboring molecules. Once the fibrin particles are aligned in an oriented fashion, secondary forces would strengthen their association.

It is believed that the clotting of fibrinogen reveals a story that has a significance beyond the borders of blood coagulation itself, and it may be an indication of how certain principles are being utilized in building biological fibers. The mechanism represented by the fibrinogen-fibrin transformation may be a common pattern in the biogenesis of a number of proteins. The enzymically altered protein displays quite different properties from those of the primary one, although it differs very little from it in molecular and chemical constitution. In the case of fibrinogen the altered protein acquires the property of spontaneous fiber formation through loss of localized charges. The concept of such relatively minor change during clotting is in agreement with all experimental data so far.

The author wishes to acknowledge the cooperation of Dr. W. R. Middlebrook, with whom much of the above work was done.

Thermal inactivation of a bacterium-bacteriophage system. MAX A. LAUFFER, ANNE BUZZELL AND DAVID TRKULA.

The inactivation at 47° of the complex, *E. coli* B and bacteriophage T2r, was studied. The investigation was undertaken to see how the thermal stability of the complex varies as the growth of the bacteriophage progresses.

Bacteriophage particles were added to very concentrated suspensions of bacteria held at 5–10° in sufficient amount to give 10^{-3} to 10^{-4} phage particles per bacterium. Thirty minutes later the suspension was diluted with medium and incubated at 37°. After various times, samples were taken and immediately heated at 47°. Plaque counts were made after various times of heating. Inactivations at 47° followed first order kinetics. The rate of destruction of complexes incubated 0 minutes at 37° was 2.7×10^{-4} reciprocal seconds, a value very much higher than the rate of destruction at 47° of either free bacteriophage or free bacteria. The rate increased approximately two-fold for each three minutes of incubation at 37° for the first 12–14 minutes. At 12 minutes, bacteriophage stable at 47° began to appear, and by 16 minutes the phage count following heating at 47° exceeded the original amount. These results are interpreted to indicate premature lysis of complexes at 47°.

JULY 28, 1953

Glycolysis and potassium-sodium exchange in rabbit red cells exposed to ethyl and n-propyl carbamate. J. W. GREEN AND F. X. WAZETER.

Washed rabbit red cells, handled under sterile conditions, were incubated in 1% NaCl phosphate-buffered solutions at pH 7.00 and 34° C. for 48 hours in concentrations of 4 ml. of packed cells per 100 ml. of NaCl solution. To the suspensions were added 100 mg% glucose and varying amounts of ethyl or propyl carbamate. Suspension samples were removed at 12-hour intervals and determinations of Na and K, hemoglobin, glucose and pH were made. It was found that 0.3 *M* ethyl and 0.1 *M* propyl carbamate exerted a marked effect on accelerating Na-K exchange; cell K loss in the former suspension attained diffusion equilibrium within 48 hours, while in the latter somewhat more slowly. With both carbamates at these concentrations, the utilization of glucose was reduced to approximately 50% of that found in the control suspensions. Lower concentrations of both carbamates had either no effect or a slight stimulating effect on glucose utilization, while 0.5 *M* ethyl and 0.3 *M* propyl carbamate proved hemolytic after 10 hours of incubation. Potassium cell losses exceeded Na cell gains with 0.3 *M* ethyl and 0.1 *M* propyl carbamate. In the presence of ethyl carbamate the total cation content of the cells was reduced by approximately 25% and with propyl carbamate by half this amount at 48 hours. Cell volume changes did not reflect these cation shifts. The action of the carbamates on these cells is regarded as non-specific and suggests gelation of the cell contents.

A study of the oxidative activity of rabbit reticulocytes. F. R. HUNTER AND ALICE S. BAKER. No abstract submitted.

The influence of metabolic factors on the distribution of sodium and potassium ions in the green alga Ulva lactuca. GEORGE T. SCOTT AND HUGH R. HAYWARD.

The cells of the green alga *Ulva lactuca*, like those of most organisms living in a high sodium-low potassium medium, accumulate potassium and maintain within a relatively low sodium level. Experimental studies relating to the following were carried out on this form: 1. The influence of light and dark on the potassium and sodium distribution. 2. The influence of iodoacetate. 3. The influence of light and temperature on the exchange rate of potassium ion.

The alga when maintained in the dark for long periods loses some potassium and gains sodium, both effects being reversed on illumination. The presence of 0.001 *M* iodoacetate in the dark causes a marked progressive loss of potassium and gain of sodium. This influence of the inhibitor is completely prevented by light. Evidence for the penetration of the inhibitor in the light is presented, in that after the material is transferred from sea water containing the inhibitor in the light to running sea water in the dark a significant loss of potassium and gain of sodium occurs. The iodoacetate effects once established can be "washed out" by transferring the alga to light and running sea water without the inhibitor. Potassium reaccumulation begins after 24 hours whereas sodium ion is secreted out of the cell to the level of the control within four hours.

The exchange of cellular for environmental potassium was measured using K^{42} under conditions of illumination and darkness and at 20° and 30° C. The data indicate a complete exchange of cellular for environmental potassium which occurs at an increased rate under conditions of illumination and increased temperature.

In the interpretation of these findings it is emphasized that energetic coupling to metabolic processes must occur in the continual movement of ions against their concentration gradients. This energy presumably is derived from the degradation of phosphoglyceric acid, an intermediate formed in the breakdown of carbohydrate reverses, except in the presence of iodoacetate, or formed by photosynthesis even though the inhibitor be present.

AUGUST 4, 1953

On the mechanism of staircase and contracture in ventricular muscle. STEPHEN HAJDU. No abstract submitted.

Conduction in non-striated muscles. C. LADD PROSSER. No abstract submitted.

Structural and in vitro diffusion characteristics of intact and "desheathed" sciatic nerves from the toad (Bufo marinus) and from the bullfrog (Rana catesbiana). ABRAHAM M. SHANES.

Recently completed studies provide conclusive proof that the peripheral connective tissue sheath (epineurium or external perineurium) of the bullfrog sciatic completely determines the kinetics of diffusion and ion exchange in the intact nerve. Thus, the slow emergence of Na^{22} into inactive solution from nerves previously equilibrated in radioactive Ringer's is practically identical with that from sheaths containing only radioactive solution in their lumen. Consequently, the sheath must be removed when the permeability characteristics of the nerve fibers are to be examined. Unfortunately, desheathed bullfrog sciatics undergo a large (40%) increase in weight over a two-hour period in Ringer's, which renders them unsuitable for quantitative work.

The desheathed toad sciatic does not change significantly in weight. Moreover, the spike amplitude and the potassium content remain constant for at least 20–24 hours. Histological sections prepared by Dr. James B. Longley demonstrate that the bullfrog sheath is a mixture of at least two structures staining differently in Masson's. Both are completely removed by the desheathing operation. In the toad nerve, however, the sheath is composed of two rela-

tively distinct structures: an outer one, which is relatively thick and takes up aniline blue, and an inner one, which is thin and stains with acid fuchsin; only the former is removed by the desheathing operation. Nevertheless, the apparent exchange (self diffusion) coefficient for the extracellular space is increased fifty-fold from the original value of about 7×10^{-7} cm.^{2} min.$^{-1}$ Although this is still 20 times smaller than in free solution, it is sufficient to permit study of the still slower ionic exchange across the membranes of the nerve fibers.

AUGUST 11, 1953

Ribonuclease activity in rat liver fractions. JAY S. ROTH.

Rat liver homogenate was separated into nuclei, mitochondria and supernatant using a modification of the technique of Hogeboom and Schneider. Cytochrome oxidase assays of nuclei and homogenates gave ratios of homogenate to nuclei activity of from 10 to 21, indicating that the nuclei were practically free of mitochondrial contamination.

Ribonuclease activity was determined in the three fractions and the original homogenate at 12 pH values from 4.47 to 8.52 by measurement of acid soluble P^{32} split from P^{32}-labeled yeast RNA. It was demonstrated that under the conditions of assay used there was no increase in inorganic P^{32}. Thus the assay was specific for ribonuclease activity.

The total ribonuclease activity in the homogenate and each fraction varied greatly depending on the pH. Mitochondria showed two distinct activity peaks both of about the same size at pH 5.8 and 7.8. Nuclei showed only one peak at 7.8. At this latter pH the specific activity of nuclei approached that of mitochondria. In the pH range 4.47 to 6.27 total activity expressed as per cent of homogenate activity was as follows: nuclei 10–17%; mitochondria 54–66%; supernatant 14–21%. In the pH range 6.8 to 7.8 the total activity of homogenate was from 20–50% of that at other pH values due probably to action of an inhibitor. Thus the activities of the fractions rose to: nuclei 90%, mitochondria 214% and supernatant 59% of homogenate activity; above pH 7.8 they dropped again. The small ribonuclease activity in nuclei is probably not due to contamination in view of the purity of the nuclei and the presence of only one activity peak.

Observations on the carbohydrate metabolism of the developing Chaetopterus egg. DWIGHT B. MCNAIR SCOTT.

A pattern of decrease of respiratory rate on fertilization and increase later was found agreeing with the findings of Whitaker. The mean rate before fertilization was 10 μ Mol O_2/hr./10^6 eggs, after fertilization 3.6 μ Mol O_2/hr./10^6 eggs, and about 5 hours later 12 μ Mol O_2/hr./10^6 eggs. The pattern of RQ values was similar to that reported by Ohman and others.

No reducing sugar was detectable in the eggs or the sea water in which they were suspended until about four hours after fertilization when reducing sugar appeared in both, only to disappear later. Glucose added to the sea water was taken up by the developing embryo at about the same rate as oxygen was consumed, after the blastula started to move.

Total carbohydrate was determined in the eggs by the anthrone method. The amount in unfertilized eggs was .015–.025 micrograms per egg, the total carbohydrate decreased in the unfertilized egg at the mean rate of 10 μ Mol/hr./10^6 eggs. After fertilization there was a sharp drop in total carbohydrate, followed by an increase. The blastulae contained a mean of .012 micrograms of total carbohydrate when they started to swim and also 10 hours later.

In 1951 S. S. Cohen found that enzymes to oxidize glucose-6-phosphate and 6-phosphogluconate could be extracted from Chaetopterus eggs both before and for twelve hours after fertilization. The measured activity of the extracts could account for the use of carbohydrate by these enzymes at the rate of 6–8 μ Mols/hr./10^6 eggs.

Thus it seems possible that carbohydrate utilized by the oxidative pathway may be the main substrate of respiratory activity in the unfertilized Chaetopterus egg.

The changes in carbohydrate after fertilization are more complicated, and no conclusions have been drawn from the experimental results.

Synthetic substrates for plasmin and thrombin. WALTER TROLL AND SOL SHERRY.

Plasmin and thrombin are usually classified as proteolytic enzymes. There seems to be little doubt that this classification is a fitting one for plasmin, an enzyme capable of splitting fibrin, fibrinogen and other proteins into peptide fragments. New evidence has been recently obtained which supports the proteolytic nature of thrombin's action. It has been shown that a peptide is split from fibrinogen by thrombin as the first step of the conversion of fibrinogen to fibrin. We have investigated esters of amino acids as possible substrates for these enzymes, and have observed that plasmin split esters of arginine and lysine, while thrombin attacked only arginine esters. The corresponding amides were also split, but to a much smaller extent. The rate of splitting of a typical substrate, such as tosylarginine methyl ester (TAMe), occurred with zero order kinetics as measured either colorimetrically by the disappearance of the Hestrine ester color, or titrimetrically by the appearance of carboxyl groups.

Three types of observations supported the view that these esters are substrates for these enzymes: 1. The proenzymes, plasminogen and prothrombin, showed no activity against these esters. Only on activation of the enzymes by known procedures did the activity appear. 2. Purification of the enzymes as measured by conventional methods led to a corresponding increase of activity against these esters. 3. The esters compete successfully with the protein substrates of the enzymes. Thus lysine ethyl ester and TAMe inhibit the proteolytic activity of plasmin on casein, and only TAMe inhibits the clotting of fibrinogen by thrombin. Other esters have no effect.

The fact that both plasmin and thrombin attack substrates of proteolytic enzymes confirms the proteolytic nature of these enzymes. Synthetic substrates offer a new quantitative tool for the measurement of these enzymes.

AUGUST 18, 1953

Studies on the mechanism of physiological dominance in Tubularia. MALCOLM S. STEINBERG.

In Tubularia very short pieces of the stem regenerate as partial bipolar hydranths, medium sized pieces (5–10 mm. long) regenerate a hydranth only at the distal end, and long pieces regenerate a hydranth at each end. Inhibition of proximal regeneration by distal regeneration is called "physiological dominance." It has been found that immediately after a stem-section is isolated, the entire cylindrical body (coenosarc) begins to stretch toward the distal end, depleting the proximal end of cells and supplementing the distal end. There being an effect of cell number upon rate of regeneration, this slows down regeneration at the proximal end, where it was already inherently slower (proximal regions normally regenerating more slowly than distal regions) and speeds up regeneration at the distal end. The distal regenerating hydranth, at about the time of its emergence, produces in active quantities an inhibitor which acts only upon regenerative processes operating before the hydranth primordium is established. The proximal end, due to its initially slower rate of regeneration and the further slowing down of that rate by the emigration of cells, is at this time in a pre-primordium stage and its regeneration is therefore completely prevented by the inhibitor. In very short stem pieces the distad movement of the coenosarc sets up a single primordium in the middle of the piece, this primordium containing all of the cells in the piece. If too few cells are present regulation of the primordium is imperfect and a partial bipolar form results. There is here no question of dominance, since that term refers to the suppression of one developmental center by another, and here there is only a single center. It is thought that proximal regeneration occurs in long stems because the inhibitor produced distally is diluted in the coelenteron to a sub-inhibiting level.

Elaboration of so-called posterior lobe hormones in the hypothalamus. WALTHER HILD.

Considerable evidence is presented that the vasopressor, antidiuretic and oxytocic hormones are not produced in the posterior pituitary. They originate by a neurosecretory process

in the neurosecretory nerve cells in the supraoptic and paraventricular nuclei in the hypothalamus, and migrate in the nerve fibers of the supraoptico-hypophyseal tract into the neurohypophysis. Here they are stored and, if necessary, released. The product of the neurosecretory cells consists of a histologically stainable carrier substance, which contains the hormones and which can be separated from the hormones by solution in several organic fluids. This concept is based on the following observations:

1) The hormone content of the supraoptic and paraventricular nuclei, the pituitary stalk and the neurohypophysis—as shown by bioassays—depends directly on the amount of stainable material present in histological sections. The hypothalamo-hypophyseal system can be depleted of neurosecretory substances (carrier substance and hormones) by dehydration of the experimental animals. After the re-establishment of water balance, a re-accumulation of both fractions is observed.

2) After interruption of the supraoptico-hypophyseal tract (neurosecretory pathway) in dehydrated animals, carrier substance and hormones accumulate in the central fiber stumps and in the nerve cells, while the peripheral fiber stumps and the neurohypophysis remain empty.

3) Bioassays of the posterior pituitary tissue grown *in vitro* show that the neurohypophysis is unable to produce hormones as claimed by several authors. The hormones transferred with the explants into the culture medium are inactivated within 7 to 10 days, at which time the nerve endings degenerate and the stainable carrier substance disappears. Neurosecretory cells grown *in vitro* for from 12 to 68 days contain small amounts of stainable neurosecretory substance. Bioassays of these cultures are still under study.

GENERAL SCIENTIFIC MEETINGS

August 26–27, 1953

Abstracts in this section (including those of Lalor Fellowship Reports) are *arranged alphabetically by authors* under the headings "Papers Read," "Papers Read By Title" and "Lalor Fellowship Reports." Author and subject references will also be found in the regular volume index (published in the December issue).

PAPERS READ

The toxic effect of a marine bacterium on Limulus and the formation of blood clots.
F. B. Bang and J. L. Frost.

While injecting large Limulus crabs with various marine bacteria, a sick crab was discovered. Blood cultures, during a 6-day period, yielded a different bacterium which formed recognizably clear, translucent colonies. These consist of small gram-negative motile rods. Injection of large crabs with 0.1 cc. of a suspension of this bacterium caused death within 6–12 hours with concentrated suspensions (about 10^8 colonies/cc.) and produced persistent (5–6 day) active infections with lower dilutions. No active infections have been produced with relatively few bacteria. One-tenth cc. of boiled suspensions repeatedly killed 12-inch Limuli and in several experiments the supernatant from such boiled suspensions also killed.

A comparison of sizes of crabs (in which 2-inch to 12-inch individuals received the same dose of boiled suspension) showed that the large crabs are more susceptible to the toxic effect. This has not been shown for the active infections.

Within 10 minutes after the injection of the bacterial suspensions, the blood withdrawn fails to clot and has very few white cells. Later, evidence of white cell breakdown is found (cellular granules free in the serum and white cell processes identified in the electron microscope). The apparent intravascular clot is not in all cases irreversible and particularly smaller crabs will yield clear fluid during the first few hours after injection and then recover. The importance of this constant phenomenon in causing death is undetermined.

Preparations of Limulus blood for electron microscopy were made by bleeding crabs onto collodion coated grids and fixing with osmium at 3 and 30 minutes. Thorough washing before fixation was necessary to obtain clear pictures.

The blood cells so prepared showed numerous long processes (average width 54–96 millimicrons, extremes up to 320 millimicrons found) extending from one to another. These same preparations showed a network of minute fibers (average width 9–18 millimicrons, extremes up to 40 millimicrons found) extending between cells. Shadowed preparations showed no detectable periodicity.

A new type of respirometer which permits continuous and automatic recording of metabolic rate. FRANK A. BROWN, JR.

A respirometer is described with which it is possible to obtain continuous records of the fluctuations in rate of O_2-consumption of either undisturbed normal, or experimentally treated, organisms over periods up to several days. The method consists essentially of a respiratory chamber containing the organism and appropriate absorbents for CO_2 and any other significant gaseous wastes such as ammonia. Directly connected to this rigid chamber is a readily collapsible plastic (Visking Saran) sack whose lumen is continuous with the respiratory chamber by a fine capillary tube. The respiratory chamber is filled with air and the plastic sack with oxygen and the whole suspended by a thread in a constant temperature bath. The thread is made to activate an appropriately sensitive scale which records continuously the weight of the "diver" on a moving strip of paper. Each milliliter of oxygen consumed by the contained organism obviously increases the weight of the "diver" by one gram. The apparatus, in addition to having the virtue of being continuously recording, is one in which the organism is maintained in an environment of essentially constant oxygen for the duration of the runs. With such an apparatus as that described in principle here, it has been possible to demonstrate persistent diurnal, tidal, and semi-lunar rhythms of metabolism in the fiddler crab, Uca.

Colliding impulse studies of the relation between longitudinal transport and afterpotential in nerve of frog and squid. KENT M. CHAPMAN.

Recent authors have presented histological and chemical evidence for longitudinal migration of materials in living nerves, for neurosecretory activity, and for liberation of substances at nerve termination. A longitudinal ion displacement persisting during the afterpotential in stimulated nerve is predicted theoretically if one assumes that action potentials are propagated as waves (*i.e.*, with constant amplitude, shape, and conduction velocity) and that cellular fluids are ohmic media. Thus the transmembrane action potential, as the time integral of the longitudinal current, represents longitudinal charge (ion) displacement; hence afterpotential following excitation implies net ion transport. The calculated displacement for frog sciatic α fibers and for squid giant axons is 10^6 to 10^7 ions/ millivolt.

Experiments were designed to test for accumulation of this material at conduction blocks produced by collisions of pairs of impulses, using external non-polarizable electrodes to measure D.C. potential between the impulse collision site and some adjacent point on the nerve surface. With this technique, accumulating material can be detected only if it measurably alters the local membrane potential.

Colliding impulse potentials accompanying periods of repetitive thyration stimulation lasting 0.1 seconds to several minutes have been studied in desheathed bullfrog sciatics (α fibers) and in partially cleaned squid giant axons; 60 cycle stimulation was used for frog; 60, 120, 180, 240 and 300 cycles stimulation and mild veratrinization for squid. Spike amplitudes were normally 10–20 mv for frog, 15–40 mv for squid; negative afterpotentials were 0.5–1 mv for frog, 0.2–2.0 for veratrinized squid axon. However, in all cases, local changes in membrane potential at the collison sites were negligible; sensitivities of the measurements were 50–100 microvolts.

One must conclude that if the predicted ion accumulation takes place, the membrane potential is insensitive to the resulting qualitative or quantitative changes.

This work was supported in part by the Grass Trust.

Surface structure limitation of fertilization. RALPH HOLT CHENEY AND ALBERT I. LANSING.

The significance of physico-chemical characteristics of the egg surface to its fertilizability is an age-old problem. *Arbacia punctulata* has provided favorable material for study of the process of fertilization. Electron microscopical examination was used to compare the normal with caffeine-treated eggs. The latter results in inhibition of fertilization. Ultramicroscopic changes occur in the finer structure of the egg border and cortical granules as the egg is transformed from the fertilizable to the non-fertilizable state. The influence of the methylated xanthine, caffeine (1:3:7 trimethyl 2:6 dioxypurine), upon this sequence of events was determined. Unfertilized eggs from a mature female (egg sampling was 95% fertilizable) were shed into sea water as control, and into molarities of *M*/5000, *M*/100, and *M*/10 caffeine-in-sea-water for a 15-minute pretreatment prior to preparation for ultra-thin sections (0.1 mu to 0.25 mu) and examination under electronic magnifications of 3,000 to 10,000 ×. Enlargements photographically gave 15,000 to 35,000 ×.

The facts *that* *M*/5000 CSW is ineffective in exercising inhibitory effects upon the ability of the unfertilized egg to become fertilized in normal time and form, *that* *M*/100 CSW retarded the time and caused abnormalities in cleavage, *and that* *M*/10 CSW completely prevented fertilization, have been reported previously by Cheney. *The fact that* the normal Arbacia egg surface possesses numerous and fairly regularly-spaced papillae which are cytoplasmic prolongations was demonstrated by Lansing, Hillier and Rosenthal in 1952. This current study of the effects of these particular molarities of caffeine via electron microscopy reveals that an increasing degree of disorganization of the cortical granule matrix and a decreasing number in surface papillae occurs with increasing concentration of caffeine until the non-fertilizable *M*/10 egg cultures show a non-papillate, smooth surface.

Oscillographic analysis of an invertebrate equilibrium organ.[1] MELVIN J. COHEN.

A continuous discharge of impulses is observed on recording from the entire statocyst nerve in the lobster *Homarus americanus* Milne-Edwards. Such a spontaneous discharge is present with the preparation oriented in any position and is composed of action potentials from individual fibers firing aperiodically at a frequency usually less than 10 per second.

Rotation about the transverse axis results in a characteristic response pattern when recording from a specific nerve bundle. Rostrum-down displacement is accompanied by a burst of activity in the acceleratory phase, followed by a marked decrease (falling below the resting level) during deceleration associated with the termination of movement. Rostrum-up displacement produces an abrupt depression of activity during acceleration followed by a burst, which rises above the previous resting level, at the termination of movement. Impulse traffic returns to approximately the original resting level within one second after cessation of movement. The discharge rate also approaches the previous resting level 0.5 seconds after constant velocity is achieved and maintained following initial acceleration in either direction. Fibers of larger diameter (as judged by spike amplitude) cease firing with rostrum-up accelerations as low as 5 degrees per second per second, while the smaller fibers decrease in frequency only with greater accelerations and cease firing as values of 30 degrees per second per second are approached.

Single fiber preparations from other bundles of the statocyst nerve have been observed to maintain a non-adapting discharge in specific positions about the transverse axis. Such static "position" units most frequently attain a maximum of 20–30 impulses per second at 45–90 degrees rostrum-up and are often inactive in the zero (normal swimming) orientation. During movement toward the region of maximum static response these fibers may attain a frequency of 50 per second, adapting to the static level within 30–60 seconds after cessation of movement.

Effect of aqueous extract of beef spleen on cells of sarcoma 37 ascites tumor. IRENE COREY DILLER.

The injection of a single dose of aqueous beef spleen extract containing 100 mg./ml. of solid materials directly into the peritoneal cavity of mice bearing ascites sarcoma 37 resulted

[1] Aided by a grant to Dr. Theodore H. Bullock from the National Institutes of Health.

in inhibition of mitosis for a period of at least 48 hours, and in some cases in complete cessation of mitosis that persisted for even longer periods. Mitotic inhibition was accompanied by a marked inflammatory reaction. Cell counts per cc. of ascites fluid were lowered and the percentage of viable tumor cells decreased, partly as the result of cessation of mitosis and necrotic change, and partly through dilution with white blood cells.

Other samples of spleen extract produced similar changes at slower rates for the same dose level, but with a considerable increase in the total number of degenerating cells.

The reduction of tumor cell population was accompanied by the formation of islands of tumor cells which presumably served as centers for the formation of solid tumors. When the tumor masses thus formed were large, no regression was observed, though survival time was considerably lengthened; and small masses were completely resorbed under continued spleen extract therapy in 30.5% of the treated animals. No reduction in ascites swelling unaccompanied by formation of small solid tumors was observed.

Feeding efficiency of bats. EDWIN GOULD.

Since Griffin has suggested that bats may use high frequency sound to capture flying insects, the rate at which bats capture insects was studied by collecting 64 *Myotis lucifugus*, 5 *Pipistrellus subflavus* and one *Eptesicus fascus* at night and weighing their stomach contents. Two hours after emergence from the roost a *Myotis* (body weight 7.6 grams) had accumulated 1.5 grams of insects. After 30 minutes flight two *Pipistrellus* contained 1.35 and 1.65 grams (25% of body weight) and one 18.9-gram *Eptesicus* caught 4.0 grams in 1.5 hours. Six *Myotis* which were collected after 45–95 minutes flight contained 0.9, 1.15, 0.91, 0.3, 0.05 and 0.95 grams. Even neglecting passage of food into the intestines this indicates a typical accumulation rate of one gram/hour under favorable conditions. Examination of 33 *Myotis* stomachs revealed remains of Lepidoptera, Coleoptera, Hymenoptera, and Diptera, but the great bulk of the material was too finely chewed for identification. Small Chironomidae (wingspread 3 mm., weight 0.2 mg.) were found, and bats were seen to pursue moths up to at least 20 mm. wingspread and 15 mg. weight. The hourly catch could thus be theoretically about 5000 Chironomidae or 66 moths, but an intermediate mixture is far more likely. Although individual moths are actively pursued it is possible that bats merely detect swarms of Chironomidae and fly through with their mouths open, employing a sort of "filter feeding." The volume of air "filtered," however, is only about 1800 liters per hour (assuming mouth area equals 1 cm^2. and flight velocity equals 5 meters/sec.), and most of the time is certainly not spent flying through dense swarms of insects. It thus seems equally possible that even individual insects as small as 3 mm. are selectively detected and pursued.

The effect of S^{35} on the development of Arbacia eggs. J. W. GREEN AND JAY S. ROTH.

Either dilute sperm or washed, unfertilized eggs of *Arbacia punctulata* were exposed to several concentrations of S^{35}. The S^{35} was prepared by evaporating carrier-free isotope solutions to dryness and taking up the S^{35} residue in sea water. The pH of such solutions was the same as that of sea water. Sperm were irradiated two hours in concentrations of 0.5, 5.0, and 50.0 microcuries of S^{35} per ml. prior to their use in the fertilization of non-irradiated eggs. Eggs were also exposed for zero, one and two hours to concentrations of 0.25, 2.5 and 25.0 microcuries of S^{35} per ml. prior to fertilization with non-irradiated sperm. After fertilization the time to 50% cleavage of the first division was determined, as well as subsequent developmental stage of the embryos between 48 and 72 hours. Irradiation of the eggs usually resulted in a small retardation in the first cleavage time with the intermediate and high concentration of S^{35} producing the longest retardations. The greatest amount of cleavage retardation occurred when eggs were fertilized and then irradiated; the least amount when the eggs were irradiated for two hours prior to fertilization. Roughly the reverse of this was observed in the subsequent development of the eggs. In some cases when control embryos were well developed plutei, the embryos in the highest isotope concentration were early gastrulae. Sperm irradiation retarded the first cleavage, even with 0.5 microcuries of S^{35}. Subsequent develop-

ment of eggs fertilized with such sperm resulted in marked retardation of plutei formation, or in skeletal abnormalities.

The effect of calcium on the proteins of sea urchin egg homogenates.[1] PAUL R. GROSS.

Several significant events follow the addition of calcium to homogenates of sea urchin eggs (the eggs having been freed of Ca prior to homogenization). Among these phenomena are viscosity increase and rupture of cytoplasmic granules. The experiments reported here represent an attempt to understand these effects at the protein level.

In the presence of Ca, some of the protein becomes insoluble in water and in *M* KCl. Study of this phenomenon over a wide range of concentrations of Ca strongly suggests that two reactions occur, one in the "physiological" range of [Ca^{++}], and the other when [Ca^{++}] is greater than 10 millimols/liter. Spectrophotometric measurements in the "physiological" range confirm a loss in protein solubility of ca. 20% and also indicate that the precipitated material is rich in nucleic acid.

By means of methods such as are used in the study of fibrinogen clotting, it is possible to demonstrate a small but consistent release of non-protein nitrogen as a result of the addition of Ca. This is consistent with previously reported proteolytic activity in the homogenates.

Electrophoresis of water and dilute salt-solution extracts of the homogenate reveals that, with low [Ca^{++}], only the slowest fraction is precipitated. Occasionally, a new, very slow boundary appears after Ca treatment.

The electrophoretic patterns are confirmed in electron micrographs of the insoluble parts of the homogenate. In the Ca-treated systems, aggregates appear. These are formed, apparently, by multipolar attractions, and are composed of spherical particles of diameter 256 ± 47 Å. Aggregates are absent from controls.

A simplified electrical method of determining the sex of sea urchins and other marine animals. ETHEL BROWNE HARVEY.

The electrical method of determining the sex of sea urchins described last year (Biol. Bull. 103: 284) has been simplified and improved. A household bell transformer (cost $1.50) is now used instead of a Variac to cut down the 110 A. C. voltage to 10 volts, the proper voltage for stimulating Arbacia. Lead electrodes have been found best; they are non-toxic and do not form bubbles as do platinum electrodes. These can be easily made from lead tubing having an outside diameter of 5 mm. with a bore of 3 mm. Short pieces of the lead tubing are placed around the ends of the copper wires from the transformer and sealed with tire tape and De Khotinsky cement to keep out the sea water. The free ends are flattened and sealed by pressure. Or short lengths of lead wire, 3 mm. diameter, in contact with the copper wire can be sealed with tire tape and De Khotinsky cement.

The two stimulating electrodes may be placed anywhere on the shell of the Arbacia, dorsal side up in sea water. Almost immediately after passing the current, the sperm or eggs exude from the five gonopores, the sperm in fine white threads and the eggs in small clumps. The emission of the sperm and eggs stops immediately when the current is cut off and begins again when the current is allowed to pass. The eggs should be removed at once to fresh sea water, and the sperm suspension kept in a refrigerator for further use. The eggs fertilize perfectly and develop normally.

By this method, males and females can be distinguished quickly with no trouble and without sacrificing an animal; and a small number of eggs can be obtained repeatedly from the same animal. The response is due to the stimulation of muscle cells in the walls of the ovary and testis. Animals containing only immature eggs do not respond.

Other animals also respond to the electrical stimulation, *e.g.* sand dollars, worms (M. J. Allen), molluscs. If a higher voltage seems to be required, a Variac should be used instead of the bell transformer.

[1] This investigation was supported by a research grant from the National Cancer Institute, National Institutes of Health, Public Health Service, administered by L. V. Heilbrunn.

Evidence for independent mechanisms regulating potassium accumulation and sodium secretion in Ulva lactuca.[1] HUGH R. HAYWARD AND GEORGE T. SCOTT.

Investigations on factors controlling sodium and potassium distribution in cells of the marine green alga *Ulva lactuca* indicate the following. Cells washed in isotonic sucrose, having lost 80–90% of their normal K^+ and Na^+ will reaccumulate K^+ linearly when returned to sea water. The rate of reaccumulation is independent of the presence of Na^+ in the medium (isotonicity in the absence of Na^+ was maintained with sucrose), indicating that K^+ accumulation need not be secondary to Na^+ secretion. K^+ reaccumulation is complete. The metabolic inhibitor phenyl urethane (10^{-3} *M*) effects a marked progressive loss of K^+ and gain of Na^+, both of which changes are completely reversible on washing the alga in running sea water under illumination.

The presence of 5×10^{-4} *M* AsO_4^{---} completely protects the cells against the K^+ loss caused by 10^{-4} *M* iodoacetate (IA^-) in the dark, while it affords no significant protection against the Na^+ gain resulting from IA^- inhibition. This again indicates that separate mechanisms are operative in the regulation of Na^+ and K^+ concentrations in these cells. The K^+ protection by AsO_4^{---} is understandable in terms of arsenolysis at the level of triose phosphate dehydrogenase, the site of IA^- inhibition.

After 20 hours in the presence of 5×10^{-4} *M* IA^- (dark) samples were transferred to fresh sea water containing 5×10^{-3} *M* AsO_4^{---}; some were illuminated, the rest maintained in darkness. At the time of transfer the cell [K^+] was 55% of the controls and the [Na^+] ca. 150%. Continued marked loss of K^+ and gain of Na^+ occurred in the IA^- samples. The combination of AsO_4^{---} and illumination produced a slight reaccumulation of cellular K^+, while in darkness AsO_4^{---} offered the cells significant protection against K^+ loss as compared with the IA^- samples. The behaviour of the AsO_4^{---} samples as regards Na^+ differed markedly in light and dark: those in the dark continued to increase in cell [Na^+], while those in the light secreted Na^+ to the control level within 12 hours.

These findings are consistent with the general association between carbohydrate metabolism and normal cation regulation. They further serve to emphasize two discreet mechanisms for controlling cellular [Na^+] and [K^+]. Finally they may suggest an important relationship between the effects of illumination (in addition to generation of carbohydrate intermediates, and perhaps associated with the reductive powers of light) and the Na^+ secreting mechanism.

The effects of various acids on the receptors of the amphibian tongue. THOMAS N. JOHNSON. See abstract in "Lalor Fellowship Reports."

Adenosine triphosphate as an energy source in cell division; pressure-temperature experiments on the cleaving eggs of Arbacia and Chaetopterus. DOUGLAS MARSLAND,[2] JOSEPH LANDAU[3] AND ARTHUR ZIMMERMAN.

The eggs of these species, immersed in a .0005 *M* sea water solution of ATP, display a very definite improvement of furrowing capacity under widely varying conditions of temperature and pressure. At atmospheric pressure, the minimum temperature at which successful furrowing can occur is reduced by two degrees centigrade, and at higher temperatures, the minimum pressure required to block the cleavage furrows is increased by approximately 500 lbs. per square inch.

These increases in the cleavage potential appear to be mediated through the effects of the ATP upon the gelational state of the cortical cytoplasm of the eggs. Centrifugal measurements of the gel strength of the Arbacia egg cortex, made at 10°, 15°, 20°, 25° and 30° C., and at pressures ranging up to 8000 lbs./in., yielded values for the ATP treated eggs which were consistently 15–20% higher than for the control specimens. In both treated and untreated eggs the gel strength increased exponentially with rising temperature and decreased

[1] Aided by a contract between the U. S. Atomic Energy Commission and Oberlin College.
[2] Supported by grant C807 from the National Cancer Institute.
[3] Damon Runyon Fellow, 1952–53.

exponentially with rising pressure, the experimental and control curves being identical, aside from the higher position of the former.

The new data appear to provide further support for the cortical gelation theory of cytokinesis. Undoubtedly the cortical protoplasm of the egg represents a type II gel (Freundlich) in which the gelation process is an endothermic reaction; and tentatively it may be assumed that energy from the high potential phosphate bonds of ATP can be utilized for sustaining the gel structure of the dividing cell.

Integration in the cardiac ganglion of Homarus.[1] DONALD M. MAYNARD.

By electrical stimulation, two cardio-accelerator fibers and one cardio-inhibitor fiber have been detected in the dorsal nerve of *Homarus*. The conduction velocity of the inhibitor is greater than that of the accelerators and is probably the largest of the three fibers which stain with methylene blue in the dorsal nerve.

Properly adjusted stimuli applied to portions of the isolated ganglion evoke a direct conducted response in only one fiber. This impulse in turn initiates activity in other units after a latent period of 0.02–0.04 seconds. This activity may be in the form and pattern of a "normal" burst, or it may consist of scattered impulses in a few units. The normal burst may be driven if the stimulus frequency is less than two per second and greater than the "spontaneous" rate of discharge. At higher frequencies, the burst may fail to follow each stimulus, coming after every other one. If a second stimulus is interposed in the latent period, the burst follows each paired stimulus at such frequencies. The latent period, which increases with increasing frequencies of stimulation, may also be shortened by the paired stimulus. Further facilitation is obtained with three or more stimuli, and driving may occur at still higher rates. The number of spikes in each burst is greatly reduced when the ganglion is so driven. If single stimuli are used at a frequency of 10 per second or more, the burst does not follow any particular stimulus, but the response rate increases with the frequency of stimulation; a stimulus frequency of 100 per second may produce an increase in burst rate of over 100%. When the unit responding directly to the stimulus is clearly a spontaneously active element, the burst often fails to follow as an entity at high frequencies and is disrupted as fibers selectively drop out with an increasing stimulus rate.

Isolation of midpieces from the sperm of Arbacia punctulata and observations on the role of the midpiece in sperm motility. ROBERT J. NEFF.

Arbacia sperm midpieces are prepared by washing fresh sperm in 50% sea water, re-suspending in distilled water and removing clumped heads and tails by centrifuging 5 minutes at 4000 RPM in a Serval high speed centrifuge. Midpieces are concentrated from the supernatant by centrifuging 10 minutes at 8000 RPM. The midpieces may be washed once in distilled water. To avoid osmotic rupture, with release and clumping of mitochondria, further washes should be done in dilute salt solutions. Mitochondria of intact sperm, isolated midpieces, and ruptured midpieces take up Janus green from dilute solutions. Mitochondria of isolated midpieces or ruptured midpieces show intense succinic dehydrogenase reactions using the cytochemical neotetrazolium reaction.

Effects of dilute sea water on fresh sperm were studied by phase contrast microscopy. In 50% sea water all midpieces become displaced to one side of the head, giving "L" shaped sperm. The tails are always attached to the head, and perhaps the midpiece, at the outer tip of the "L". In 30% sea water, the heads first form crescents around the midpieces, then swell to rounded structures. Midpieces only begin swelling in 10–20% sea water, forming signet ringlike structures due to the two or more mitochondria attached to their periphery. In dilutions greater than 50%, the tail usually bends under with the midpiece and points in the same direction as the tip of the head. In 0.5 *M* KCl the midpiece and tail bend under, and eventually the tail forms a circle around the entire sperm. Movement is slowed in 30–40% sea water.

[1] Aided by a grant from the Grass Trust for Research in Neurophysiology and in part by a grant to Theodore H. Bullock from the National Institutes of Health.

In favorable preparations, the midpiece is observed to execute rapid metronome-like movements in the absence of distal tail movement.

These observations indicate that *Arbacia* sperm is bilaterally symmetrical and suggest that most, if not all, of normal sperm movement originates in the midpiece with the tail acting as a sculling apparatus.

The motion of echinochrome granules in the unfertilized egg of Arbacia punctulata. ARTHUR K. PARPART.

In addition to a slight amount of Brownian motion the echinochrome granules (they are spheres ranging in diameter from 0.5 to 2 μ) of unfertilized eggs of Arbacia also show a straight-line translatory motion which may amount to as much as 5 μ per second at 24° C. This translatory motion is markedly slowed when the temperature is lowered to 10° C., while the Brownian motion appears unaffected. This is true of these granules whether near the surface or deep in the cytoplasm. Calcium ions added iososmotically to sea water do not affect this motion until the egg is killed by too high a concentration of calcium, at which point the whole cytoplasm gels. Sodium fluoride (0.01 *M*) and idoacetic acid (0.001 *M*) do not alter the translatory motion of these granules. The echinochrome granules of eggs whose cytoplasm has been partly separated by centrifuging do not show the translatory motion nor does recovery of the motion occur. These observations were made by T-V microscopy at 4500 × and recorded by motion pictures. It is suggested that the echinochrome granule may have several temporary and contractile fibers attaching it to the surface of the egg and the shortening of these fibers may lead to their zigzag motion thru the cytoplasm. The translatory motion of these granules is frequently across the stream of the marked cytoplasmic streaming evident in the unfertilized egg.

Cation interrelationships at the Fundulus ectoderm. D. R. SHANKLIN.

Using dechorionated 5–7 day *Fundulus heteroclitus* embryos as the living material, cation relationships were studied with the radiotracer elements: Na^{22}, Na^{24}, K^{42}, and Ca^{45}. The solutions, made to conform to an analysis of the Woods Hole supply sea water, contained these ions and magnesium in monary, binary, ternary, and quaternary combinations, with chloride as the sole anion. The exchangeable ions were found to have been equilibrated by ten hours; thus this was chosen as the end point. The major findings are: (1) In binary states, with the uptake from monary media as the base, calcium and sodium are mutually inhibitive; potassium and calcium are mutually inhibitive; sodium facilitates potassium, and potassium inhibits sodium; magnesium inhibits sodium and potassium, and facilitates calcium. (2) In ternary mixtures, *e.g.*, CaNaK, all three are inhibited, in a degree predictable by the summation of binary effects. (3) Magnesium plays an exotic role, for the union of both bivalent ions with either univalent ion is distinctly favorable to the uptake of any of the three tracer elements, when compared to the union of either bivalent ion separately, including the binary solution CaMg. These effects of magnesium are not additively predictable from binary action. (4) The complementary consideration of absence of an ion yields a corresponding picture. (5) In summation effects to the quaternary state, the effects are additive for sodium and calcium, but not for potassium; indeed, potassium is singularly facilitated above that predictable from binary and ternary actions. (6) Sea water was run correlative to the quaternary solution, and anion effects, facilitative for sodium and potassium, and inhibitive for calcium, were found. (7) The activity of the ions in these media is in reverse order of their concentrations, which is $Na > Mg > K > Ca$, the activity of magnesium a predicted value from the data.

The effect of rapid shifts of sodium and potassium on the membrane potential of muscle fibers of the spider crab, Libinia emarginata. WILLIAM K. STEPHENSON.

If leg muscles of the spider crab, *Libinia emarginata*, are isolated in sea water, the fibers loose K^+ and take up Na^+ and Cl^- at a rapid rate. From original concentrations of 93 mM./Kg. wet weight (mM.) for Na^+ and 115 mM. for K^+, the cation balance shifts to about 250 mM. of Na^+ and 50 mM. of K^+ within 1 hr.; each process having a half-time of 15 minutes.

Chloride increases somewhat more slowly from 80 mM. to 160 mM., with a half-time of 20 minutes. Muscle preparations do not change weight during the first 30 minutes of soaking in sea water, and during the next 90 minutes gain only 10% over their original wet weights. Thus, the large increase in Na^+ and Cl^- concentrations is not primarily due to a loss of water to the hypertonic medium.

Using the glass micro-electrode technique of Ling and Gerard, a membrane potential of 69.2 ± 1.3 (S.E.) mV. was obtained for muscle fiber preparations isolated in sea water. A study of the time course of potential change following immersion of the muscle in sea water reveals that the potential rises to a constant level within 10 minutes and thereafter does not change appreciably for two hours. Crab muscle, therefore, maintains a constant potential while very large shifts of Na^+, K^+, and Cl^- are taking place across the fiber surface.

In cases of rapid ion movement, the membrane potential is independent of any simple ion gradient, and thus the familiar Nernst equation does not apply. Further, the mechanism responsible for maintaining the membrane potential is effective in the face of a high permeability to Na^+ and K^+, and must be operating with a large enough safety factor to prevent these ions from "shorting out" the potential as they diffuse inward in response to their electrochemical gradients.

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PAPERS READ BY TITLE

Osmoregulating capacity in populations occurring in different salinities. JOHN D. ANDERSON AND C. L. PROSSER.

Populations of blue crabs and quahogs from different salinities were tested for differences in osmoregulation. Osmoconcentration was measured by a melting point method and expressed in equivalent normality of NaCl. Average normal concentrations at low tide collections are:

	Location	Water osmoconcentration	Blood osmoconcentration
Crabs	Pettaquamscutt River		
	Near mouth	0.43 (N NaCl)	0.46 (N NaCl)
	Head of lake	0.10	0.38
Quahogs	Great Pond, Falmouth		
	Near mouth	0.40	0.44
	Perch Pond	0.20	0.33

Animals from high and low salinities were put under stress by placing them directly into a series of dilutions of sea water. Survival in concentrations lower than 10% S. W. was significantly greater for crabs from low than from high salinity. The crab blood concentration was regulated over a wide range of external concentration, but regulation failed in high salinity crabs at about 0.1 N NaCl equivalent and in low salinity crabs at 0.04 N NaCl equivalent. In 100% S. W. (0.51 N NaCl equivalent) blood was hypotonic (0.46 N NaCl). After a week in 100% S. W. the blood concentrations and critical external concentrations for both groups were similar. In contrast to Callinectes, quahogs and marsh mussels adjust their blood to external concentration as long as they pump water but Venus failed to pump below about 50% S. W. and Modiolus failed below 35% S. W. Quahogs from dilute regions tended to pump at lower concentrations than from concentrated water. It appears that natural populations of Callinectes and Venus from different minimal salinities differ in state of osmotic adaptation; stress tests applied thus far indicate these differences as phenotypic.

On the hydrolysis and transpeptidation of glutathione in marine forms. ERIC G. BALL, OCTAVIA COOPER AND EDITH C. CLARKE.

A manometric method has been developed to follow the reactions whereby glutathione undergoes either enzymatic hydrolysis or transpeptidation at its γ-glutamyl peptide linkage. It is based upon the more marked ability of one product of these reactions, cysteinylglycine,

to coordinate with ferrous iron and carbon monoxide than is shown by glutathione itself. The reactions are carried out in an atmosphere of CO at 30° C. and pH 9.0. The various tissues studied have been homogenized usually in borate buffer. Fish kidney shows the most activity of all marine tissues studied. Results are expressed in μmols of CO uptake per 100 mg. of tissue per hour. (Theoretically 1 μmol of CO is taken up for each μmol of glutathione reacting.) Values for the kidney of the following species are as follows: scup, 3.51; sea bass, 2.51; tautog, 1.97; dogfish, 1.70; butterfish, 1.39; toadfish, 0.53. In contrast to mammalian kidney preparations (which are 10–20 times as active), no significant difference in rate is observed in the presence or absence of alanine. Whether this indicates absence of transpeptidation in fish kidneys remains to be determined. The spleen of all these fishes shows a low activity (0.37–0.03). The heart, brain, liver, gonads, gills, intestine and swim bladder red gland of fish show no activity. No activity could be found in the eggs and sperm of *Arbacia punctulata* or various organs of *Venus mercenaria* and *Busycon canaliculatum*. The distribution pattern of the enzyme is thus not consistent with the hypothesis that it is involved in protein synthesis. If the enzyme is important to kidney function, then the assignment to it of a role in absorptive processes seems favored by a consideration of the relation of enzyme content to the structure and function of the kidneys studied (*e.g.*, toad fish).

A further study of the effects of centrifugation and low temperature on the development of Fundulus heteroclitus.[1] LOY J. BARNES.

It has previously been shown that centrifugation of *Fundulus* eggs shortly after fertilization at low forces of gravity produces total or partial duplications in a low percentage of cases. The present experiments consisted in varying the time of treatment and the force of gravity employed. Eggs were handled as previously described. Centrifugation of eggs beginning from 10 to 90 minutes after fertilization confirmed previous observations that a "sensitive period" for the production of duplications occurs within the first 30 minutes after fertilization when the blastodisc is forming. Doubling the force previously used (to ca. 600 g.) did not produce significant increase in the occurrence of duplications. A combination of centrifugation (at 300 and 600 g.) and low temperature (4° C.), the latter applied during the first 24 hours after fertilization, did not produce duplications in higher frequency than occurred in some batches of eggs which were only centrifuged. No general conclusions can be drawn as to a method for producing duplication by altering gravitational force at this time, but the types of abnormalities found (in addition to true duplication), including reduced and defective head structures, mesodermal deficiencies and rudimentary "parasitic twin" structures, suggest that any agent capable of separating a presumptive organization center produces effects commensurate with the degree of redistribution of ooplasmic materials.

Elastic and ionic studies with the squid giant axone. L. L. BOYARSKY.

The static elastic modulus of cleaned squid axones was determined tensiometrically. The order of magnitude of Young's modulus was 10^9 dynes/cm.^{2} This value is attributed to the connective tissue components since crushing a small central region does not affect the stress-strain curve. The breaking strength of normal axones was 4.9 ± 0.7 grams. The magnitude of the action potential obtained with fixed external electrodes was independent of stress up to break. Yield was not plastic and occurred at strains of about 30%. Conduction time and rate of rise of spike were also unchanged up to these extensions. Similar studies were carried out with nerves placed in artificial sea waters. Control studies showed that with unstretched nerve at *in situ* lengths, magnesium will substitute for calcium in repressing spontaneous activity. Nerves survived equally well at 50 mM calcium or magnesium concentrations, the other constituents being at the usual concentrations found in sea water. At 15–20 mM calcium or magnesium, the other ion having been excluded, spontaneous activity began. Nerves survived for hours at these concentrations. The magnitude or rise time of nerves kept in such low-calcium solutions was unaffected by stretch. These results suggest the presence of isolated areas in the membrane which permit ionic transfer during propagation. During stretch these areas are unaltered.

[1] Supported by funds from U. S. Public Health Service and the Office of Naval Research.

Diffusion coefficient of hemerythrin. ARTHUR S. BRILL AND JOHN M. OLSON.

Three-tenths per cent solutions of hemerythrin from the marine annelid, *Phascolosoma gouldii*, were prepared in 0.1 *M* phosphate buffer of pH 7.0. Diffusion of hemerythrin from solution into solvent was recorded in a Perkin-Elmer portable Tiselius electrophoresis apparatus.

By the Longsworth scanning method, two oxyhemerythrin boundaries at 22° C. were photographed three times in a period of 36 hours. During this period a gradual transition to methemerythrin was observed. Similarly two methemerythrin boundaries at 20° C. were photographed three times in a period of 20 hours. In this case the solutions had become noticeably cloudy by the end of the observation period.

For each boundary a diffusion coefficient value was calculated by the maximum ordinate-area method. All values were converted to the standard conditions of $t = 20°$ C. and solvent viscosity equal to the viscosity of water. The values from the oxyhemerythrin boundaries are 5.90×10^{-7} and 5.27×10^{-7} cm.^{2}/sec. The values from the methemerythrin boundaries are 5.87×10^{-7} and 6.27×10^{-7} cm.^{2}/sec. The average of the four values is 5.8×10^{-7} cm.^{2}/sec.

Temperature independence of the frequency of the endogenous tidal rhythmicity of the fiddler crab, Uca pugnax. FRANK A. BROWN, JR., MIRIAM F. BENNETT AND MURIEL I. SANDEEN.

The influence of temperature upon the physiological mechanism determining the frequency of the tidal rhythm was investigated. This was done by utilizing the fact that the average stage of the melanophores in legs isolated from normal *Uca pugnax* one-half hour following their autotomy reflects directly the tidal rhythm within the animal by being at a minimum in legs removed at the time of day of high tide in the region of collection of the animals. A large number of crabs was divided into three lots two to three hours preceding the time of high tide. One lot was maintained in sea water at 30–32° C., a second lot at 12–14° C., and the third lot at room temperature. Legs were caused to be autotomized from animals removed from each of the three lots at hourly intervals over a 5- or 6-hour period. The fact that the three lots arrived synchronously at the condition of minimum dispersion in the isolated legs, coinciding accurately with the actual time of high tide, clearly demonstrated that there was no influence of temperature upon the frequency of the tidal rhythm over the 18 to 20° temperature range thus investigated.

Endogenously regulated diurnal and tidal rhythms in metabolic rate in Uca pugnax. FRANK A. BROWN, JR., MIRIAM F. BENNETT AND H. MARGUERITE WEBB.

The O_2-consumption of normal *Uca pugnax* was determined continuously over a lunar period in automatic, continuous-recording respirometers. The crabs were collected at intervals and several run concurrently in individual respirometers at constant temperature and illumination for 2 to 4 days each. The daily respiratory-rate pattern varied systematically from day to day, clearly exhibiting evidences of persistent diurnal and tidal rhythms. The form of the diurnal rhythm was ascertained by averaging the respiratory rate for each hour of the day for a whole 15-day tidal period. The tidal rhythm was thereby randomized in that all phases of a tidal cycle occurred at each time of day. The diurnal cycle exhibited increasing metabolic rate from midnight to about 6 A.M., decreasing to a minimum about noon, then increasing to a second maximum about 10 P.M., with a second but lesser minimum occurring about midnight. Moving consecutive daily respiratory patterns backwards at an average rate of about 50 minutes per day, thereby keeping the tidal cycles in phase and utilizing in this manner 29 days of data, the diurnal rhythm was randomized, and a clear tidal rhythm of metabolic rate became evident. The maximum O_2-consumption occurred about low tide, and minimum rate about high tide. The overall average rate of O_2-consumption was about 50 ml./kg./hr. and in both the diurnal and the tidal cycles, an average of about a 60% increase was involved in passing from minimum to maximum rates.

An antimitotic substance in the ovary of the common puffer, Sphaeroides maculatus.[1] PIERRE COUILLARD.

The ovaries of the starfish and of other invertebrates contain antimitotic substances which apparently owe their activity to their heparin-like nature (Heilbrunn, Wilson and Harding, 1951). Among the vertebrates, fishes of the family Tetraodontidae contain in their ripe ovaries a poison, tetrodotoxin, whose empirical formula, molecular size and action on the frog heart suggest a relation to the breakdown product of heparin found last year by Chaet. Accordingly, an attempt was made to discover if ovary extracts from a local tetrodont fish (*Sphaeroides maculatus*) had an antimitotic action and if, moreover, the physiological effects of such an extract could be related to the presence of heparin-like substances. Unfortunately only spent ovaries were available; they contain little or no tetrodotoxin.

Neutral sea-water extracts of such ovaries will, in low concentration, induce abortive cleavage of Chaetopterus eggs previously fertilized in sea-water; a well-defined, almost equational furrow forms which soon regresses. Phase contrast shows, however, that karyokinesis proceeds normally in these eggs as an increasing number of amphiasters can be observed. If, from threshold concentrations of the extract, the eggs are transferred into sea-water, they divide almost at once into a corresponding number of blastomeres. Higher concentrations completely and irreversibly inhibit cleavage; centrifugation experiments show that in these cases, mitotic gelation is prevented. The extracts have little or no effect on the clam heart. Preliminary experiments indicate that the active principle of the extracts is not readily dialyzable.

Acid hydrolysates of the ovary contain considerable hexosamine (about 2 mg. per gm. of fresh tissue); extraction by the phenol partition technique (for heparin), yielded a metachromatic, anticoagulent substance; this presumed heparin has accounted so far, for 30% of the total hexosamine.

Thus it seems that, besides tetrodotoxin (still to be investigated), the puffer ovary contains a large heparin-like molecule, presumably responsible for the antimitotic action of ovarian extracts.

Clotting of the perivisceral fluid of the sand dollar, Echinarachnius parma. ERIC DAVIDSON.

Clotting occurs after tissue injury. The clot is formed exclusively from cellular elements; the extracellular fluid does not clot. Cells separated from the plasma by centrifugation and re-suspended alone in sea water will aggregate to form a clot, though slowly. The separated plasma does not gel.

Red and white amebocytes are present in the perivisceral fluid. Red cells do not break down, lose color, or shape, in a clot; white cells are the clotting agents. In clotting, the exterior processes of the white cells contract to form fibers, which interlock with those of other cells.

No pigment granule breakdown (such as occurs in Arbacia) accompanies clotting.

The tendency of the perivisceral fluid to clot around foreign particles (such as fine washed sand) can be utilized in establishing an end point in determination of clotting time.

When tissues are injured, a tissue factor is released which is essential for the clotting process. Besides this tissue factor, free calcium is also necessary for clotting. Calcium is involved in white cell breakdown. After white cell breakdown, calcium absence does not inhibit clot formation, but previous removal of calcium prevents breakdown and clotting; 0.35 *M* Na citrate was used to remove free calcium.

Tissue factor is released in the absence of calcium though it is then ineffective. If tissue factor is absent, then calcium alone cannot effectively induce clotting. Cells separated from tissue factor in the plasma by centrifugation clot eventually; either because they secrete tissue factor or because the original tissue factor is present, adsorbed to the surfaces of the cells. Such clots are abnormally thin.

[1] This investigation was supported by a research grant from the National Cancer Institute, National Institutes of Health, Public Health Service, administered by L. V. Heilbrunn.

Some properties of the mitotic inhibitor in starfish ovary extracts.[1] ARNOLD DUNN.

Heilbrunn, Wilson and Harding (1951) showed that acid sea water extracts of starfish ovary can prevent cleavage in marine eggs and that this inhibition is accompanied by a liquefaction of the cytoplasm of these eggs.

Heilbrunn, Chaet, Dunn and Wilson (1952) reported that after ammonium sulfate fractionation of the extract, the active substance remains with the globulin fraction. If extracts were placed in cellulose sacs and dialyzed against sea water, the active substance passed through the sac. Since the activity of the antimitotic substance was destroyed by periodate and since crude extracts were metachromatic when tested with toluidine blue, the indication was that the antimitotic substance might be a polysaccharide similar to heparin.

If a solution of the globulin fraction is dialyzed against 0.3 N sodium chloride for 48 hours or longer and the dialysate chromatogrammed on paper, the chromatogram shows the presence of carbohydrate and the absence of proteins, amino acids, and nucleic acids. The dialysate is metachromatic; this metachromasia disappears when globulin proteins are added. Apparently the active substance may be combined with protein as a mucoprotein which can be disassociated by dialysis. To test this hypothesis, proteins of the crude extract were separated by paper electrophoresis. Protein polysaccharide complexes were detected by the method of Köiw and Grönwall. These tests show that polysaccharides migrate with the globulin peak. If the globulin fraction is digested with trypsin and the resulting suspension treated to remove trypsin and remaining proteins, polysaccharide can be precipitated with alcohol. This polysaccharide prevents cell division in Chaetopterus eggs. The inhibition can be reversed by returning the eggs to sea water. The polysaccharide also prevents the clotting of sheep plasma and is metachromatic. Thus it seems clear that the antimitotic substance is similar to heparin; within the cell it may be bound to globulin proteins.

Effects of X-radiation on oxidative systems of Tetrahymena pyriformis. HERBERT J. EICHEL AND JAY S. ROTH.

Tetrahymena pyriformis S. grown in proteose-peptone for 72 hours, were used to prepare cell concentrates or cell-free homogenates. Cell respiration, and the activity of several enzymes in the homogenates were determined after irradiation with 300,000 or 600,000 r. Succinic (1), malic (2), and glutamic (3) dehydrogenases were assayed manometrically and catalase (4) titrametrically in homogenates prepared from irradiated cells and in homogenates which were irradiated directly. With the latter, the following changes were observed at the end of 80 minutes post-irradiation: at 300,000 r, (1) -40%; at 600,000 r, (1) -45, (2) -50, and (3) -20%. Immediately after irradiating homogenates (4) decreased 25%, while 1–2 hours later the activity increased to that of controls. After 24 hours, (4) was 50% more active in the irradiated homogenates than in the controls. With cells irradiated at 300,000 r and then homogenized, (1) decreased 9% 80–140 minutes following exposure. In homogenates prepared from cells irradiated at 600,000 r, (4) was unchanged 2 hours after exposure.

At 300,000 r respiration of concentrated cell suspensions was only slightly affected, but at 600,000 r it was decreased by 17, 40, and 53% at 30, 80, and 140 minutes, respectively, after irradiation. 1,2-transcyclopentanedicarboxylic acid, and 2,6-diaminopurine, which have been reported to alter cell permeability, protected irradiated cells under some conditions. The oxidation of L-phenylalanine and acetate by irradiated and control cells was also studied. Oxidation of phenylalanine by irradiated cells was depressed but irradiated organisms showed greatly increased oxidation of acetate. Thus, with 300,000 r at 30, 80, and 140 minutes post-irradiation, respiration of irradiated cells diluted 1:1 with distilled water was depressed by 34, 38, and 43%, respectively, while with irradiated cells added to acetate the corresponding changes were $+2$, -13, and -32%. As a result of the increase in acetate oxidation by irradiated cells, the net effect was a significant protective action on respiration achieved by treatment *after* irradiation, one of the few examples of such action.

[1] This investigation was supported by a research grant from the National Cancer Institute, National Institutes of Health, Public Health Service, administered by L. V. Heilbrunn.

A study of some proteolytic factors in the unfertilized Chaetopterus egg.[1] LESTER GOLDSTEIN AND NORMA O. GOLDSTEIN.

In a recent paper (Goldstein, 1953) it was postulated that a protease was involved in the breakdown of the germinal vesicle nucleus of the egg of *Chaetopterus pergamentaceus*. Consequently, an attempt was made to isolate such an enzyme from unfertilized eggs, and to study its behavior and the relationship of such behavior to the dissolution of the nuclear membrane.

Generally, the eggs in sea water were freeze-dried, the resultant powder re-suspended in 0.035 *M* sodium citrate, and the suspension homogenized. These preparations were tested for proteolytic activity by a modification of the Anson technique.

Apparently there are at least two different proteolytic factors present. One, with a relatively high activity, has a pH optimum of approximately 2.8 if a hemoglobin substrate for peptic digestion (Anson) is used; with a hemoglobin substrate for tryptic digestion (Anson) the pH optimum is approximately 4.0. The other factor, which has relatively low activity, apparently has a broad pH optimum, about pH 7–9, and appears to behave differently with each of the 4 substrates employed (the two mentioned above, with and without Ca).

In the paper mentioned above it was suggested that the protease involved in nuclear breakdown would show a rise in activity following liberation of the eggs from the ovary, and that after the breakdown had occurred the activity would fall off—as a result of some protective "brake" mechanism. Analyses of homogenates prepared at various times after the eggs were shed indicate that only the protease with the acid pH maximum (especially when tested on substrates prepared for tryptic digestion) behaves in the postulated manner—the other protease does not. The picture is not perfect because adequate data could not be obtained on the early stages, *i.e.*, immediately following liberation of the eggs.

Photosynthetic activity of phycobilans in some red and blue-green algae. F. T. HAXO AND PHYLLIS S. NORRIS.

Photosynthetic action spectra of some Woods Hole algae have been determined over the past several summers, employing the polarographic method for oxygen determination applied by Haxo and Blinks to Pacific Coast marine algae.

Photosynthetic activity in the bright red, deep-growing red alga, *Grinnellia americana*, follows closely thallus absorption in the middle portion of the visible spectrum where absorption is due almost exclusively to r-phycoerythrin, but deviates downward from thallus absorption in the red and blue portions of the spectrum where chlorophyll *a* absorbs strongly.

Vegetative portions of *Porphyra umbilicalus* thalli (olivaceous in color) display action and absorption spectra rather like those reported earlier for the West Coast species, *P. perforata*. Somewhat similar results but with evidence of greater phycocyanin activity have been obtained for the bluish-green colored, fresh-water red alga, *Batrachospermum sirodotii*. The absorption spectrum of the yellowish male margins of *P. umbilicalus* resembles that of green algae, the absorption being much higher at 675 mμ than at 560 mμ, apparently due to a considerably decreased phycerythrin/chlorophyll ratio. The corresponding curve for photosynthetic activity showed a definite, but minor, peak at 675 mμ; however, maximum activity was in green light.

Action spectra of *Porphyridium cruentum* (from cultures), a red alga which contains a single peaked phycoerythrin, showed maximum activity in green light and very little activity in regions of strong chlorophyll absorption. Similar results were obtained with *Phormidium ectocarpii*, a red-colored, marine blue-green alga, isolated in unialgal culture at Woods Hole.

In general these results confirm and extend earlier reports that the phycochromoproteins are the major active light absorbers in the photosynthesis of the Rhodophyta (and possibly the Cyanophyta), the light absorbed by chlorophyll *a* being utilized with relatively lower efficiency.

[1] This investigation was supported in part by a research grant from the National Cancer Institute, National Institutes of Health, Public Health Service, administered by L. V. Heilbrunn.

The action of parthenogenetic agents on the interior protoplasm of the Chaetopterus egg.[1] L. V. HEILBRUNN AND WALTER L. WILSON.

For some time now it has been our belief that stimulation in general, and stimulation to mitosis in particular, involve a release of calcium from the outer cortex of the cell and a clotting or gelation in the cell interior. Evidence for the first part of this thesis was presented last year (Wilson and Heilbrunn, 1952) for the egg of the marine worm Chaetopterus. Using the same egg, we have now been able to show that all the agents that incite the cell to divide do actually cause a sharp increase in the viscosity of the interior protoplasm. We have studied the effect of hypertonic solutions, of acid and alkali, of isotonic potassium chloride solutions, of ultraviolet radiation, and of heat and cold. All these agents induce cell division; and not infrequently the treated eggs go on to produce motile larvae. During or after exposure, tests of protoplasmic viscosity were made with the centrifuge method. In all cases, exposures which cause the egg cells to divide also produce a gelation in the protoplasm. Moreover, there is a rather close correlation between the percentage of cleavage produced and the percentage of eggs showing gelation. If, however, the gelation is too intense, death rather than cell division may ensue.

The results provide additional evidence for the calcium release theory of excitation, and on the basis of this theory it is possible to interpret the action of the many diverse agents that induce cells to divide.

Antimitotic substances present in ovaries.[1] L. V. HEILBRUNN, WALTER L. WILSON AND MURIEL LIPPMAN.

In many species of animals, eggs remain for long times in the ovary without undergoing mitotic division; then as soon as they leave the ovary, they divide. This is an indication that antimitotic substances may be present in ovaries and indeed our earlier work has shown that the starfish ovary contains a very potent antimitotic substance and that such substances are also found in the ovaries of clams and sea urchins. In the present study, we have extracted the ovaries of various animals with acidified sea water, then neutralized the extract and tested its effect on cell division in the egg of the worm Chaetopterus. We have found antimitotic substances in the ovaries of a wide variety of animals, including the lobster, 7 species of fishes, a frog, a salamander, and 3 species of mammals. The potency of these antimitotic substances varies widely, but all of them act like starfish ovary extract in keeping the protoplasm fluid and preventing mitotic gelation. It must not be thought that the ovary is unique in containing antimitotic substances; indeed we believe that it is a characteristic of all types of cells to contain heparin-like antimitotic substances. However, in general, whenever we compared the potency of an ovarian extract with the potency of an extract from some other organ or tissue, as for example liver or muscle, we found that the extracts obtained from the ovary were more potent in their antimitotic effect.

Use of autotomized legs in determining the phases of the tidal rhythm in Uca pugnax. MARGARET N. HINES.

Earlier methods of determining the phases of the endogenous tidal rhythm of the fiddler crab have involved the determination of the phases of the 15-day rhythm which results from the simultaneous possession of both persistent diurnal and tidal rhythms. The method to be described enables the phase to be determined in a few hours of a single day. To do this, the crabs are kept in pans in the laboratory and at intervals through the day sample animals are induced to autotomize their legs. The melanophores of the legs at the time of their isolation typically have their pigment dispersed. Following isolation the pigment tends to concentrate.

[1] This investigation was supported by a research grant from the National Cancer Institute, National Institutes of Health, Public Health Service, administered by L. V. Heilbrunn.

The chromatophore stage observed at 30 minutes has been found to vary in such a manner that the stage is at a minimum at the time of day of high tide in the place where the animals had been collected.

Physiological studies of centrifuged normal and carcinogen-treated Tetrahymena. MIRIAM I. JACOB.

Tetrahymena pyriformis (strain E) were serially transferred for more than 600 generations through media containing various carcinogens in 2% proteose peptone, 0.2% yeast extract, and 1% polyoxyethylene-40-sorbitol cottonseed oil (Atlas Compound G-1394). The carcinogens were dissolved in G-1394 and then added to proteose peptone media. Addition of the solubilizer to the media caused a significant increase in the period of logarithmic growth and in the total number of organisms, compared to a control medium. Growth rate and number of organisms was considerably reduced in media containing 100 μ gm./ml. of 4-aminoazobenzene, 4-monomethylaminoazobenzene, or 4-dimethylaminoazobenzene. Serial transfers for about 300 generations through 4-aminoazobenzene and 4-monomethylaminoazobenzene caused decrease in size, and alteration in shape, of *Tetrahymena*. Organisms grown in azo dyes contained granules and globules which were yellow, compared to similar, colorless, organelles in control *Tetrahymena*. Centrifugation at 7000 g for 90 minutes in a gradient medium composed of 20% gum arabic and distilled water resulted in elongation, stratification, and fragmenting of the cells into light and heavy halves. *Tetrahymena* grown in proteose peptone have a higher density than organisms grown in media containing G-1394, with or without carcinogens. Cells from 7 day old cultures, although still in the log phase of growth were more prone to fragment than those from 72 hour cultures. Fixation and staining of centrifuged *Tetrahymena* indicated the following distribution of intracellular components. The nucleus was displaced to the heavy half. The light half was filled with granules and globules which occurred in larger numbers in media containing G-1394, then in control proteose peptone medium. Treatment with OsO_4 indicated that lipid material was concentrated in the light half. The light half of stratified whole cells grown in media with azo dyes was bright yellow. This suggests that the azo dyes are associated with the lipoidal components of the cell.

Structure of the axoplasm as revealed by spontaneously-formed vesicles. CHIEN-YUAN KAO.

In freshly isolated giant axons of the squid, there are numerous well-formed vesicles in the peripheral layer of the axoplasm. These vesicles are oval, about 30 μ in width and 50–150 μ in length, tending to occur in chains of four to six individual components or in conglomerates. They are formed from filamentous structures which are visible within a few minutes after dissection. Nodose swellings appear in these filaments which gradually enlarge and fuse into distinct oval vesicles. The vesicles are transparent and have a faint pink tinge. They are structureless except for an investing film which has a slightly higher refractive index than the axoplasm.

When the axons remain in the gel state, the long axes of the filamentous structures and the vesicles are always arranged parallel to the long axis of the nerve fiber. In this state, the vesicles do not float upwards in vertically placed nerves, nor can they be moved about by a micropipette. When the axoplasm becomes a sol after the nerve has been kept in sea water, or as the result of injections of divalent cations Ca^{++}, Mg^{++}, Sr^{++}, and Ba^{++}, these vesicles round up into spheres and can be moved about by the tip of a micropipette or a micro-stream of injected fluid. These observations confirm previous studies with micro-injected oil drops that there is a linear organization in the fresh axoplasm and that the divalent cations have an unusual liquifying effect on axoplasm, in contradistinction to their coagulating action on other types of protoplasm.

The vesicles maintain their form in axoplasm extruded from the nerve, but disappear on treatment with alcohol, probably because the investing film is alcohol-soluble. The formation

by progressive swelling, the slightly higher refractive index and the alcohol solubility of the investing film suggest that the vesicles may represent intra-axonal myelin forms comparable to those observed in electron micrographs of vertebrate nerves.

The effect of a sulfide-blocking reagent on the oxygenation of hemerythrin. I. M. KLOTZ, S. RAPAPORT AND E. V. H. ROSENBERG.

It has been shown by Riggs that *p*-chloromercuribenzoate affects the interactions among the heme groups of hemoglobin but does not change the total uptake of oxygen. Markedly different results have been obtained in a study of the effect of the mercurial salyrganic acid upon oxyhemerythrin.

Hemerythrin from *Phascolosoma gouldii*, crystallized by dialysis in the cold against 20% ethanol, was dissolved in borate buffer of pH 8.1 and ionic strength 0.08. Addition of salyrganic acid (Hg/Fe ratio approximately ½) caused a rapid loss of oxygen by the pigment as judged by the disappearance of color. Oxyhemerythrin shows absorption maxima, in addition to the protein band at 280 mμ, at 330 mμ and 500 mμ. In the presence of mercurial an additional peak appears at 375 mμ, whereas that at 500 mμ disappears. Either of these latter peaks may be used to follow the effect of the mercurial. At room temperature the reaction took about one-half hour.

The primary affinity of the mercurial is for sulfide groups. It seems, therefore, that the iron in hemerythrin must be bound to the protein at least in part through a sulfide linkage, and that the scission of the S-Fe bond results in the release of bound oxygen. Since there are approximately three Fe atoms per mole of O_2 in fully oxygenated hemerythrin, it was of interest to determine quantitatively the minimum amount of mercurial required to deoxygenate the protein. Titration experiments with salyrganic acid were carried out at approximately 3° C. to minimize the spontaneous conversion of hemerythrin to methemerythrin. The results indicate that one mole of mercurial per three iron atoms completely deoxygenates hemerythrin. Thus there seems to be only one sulfide group per three iron atoms and it appears possible that only one of the iron atoms is actually linked to O_2.

Hexokinase and isomerase activity in eggs of the sea urchin, Arbacia punctulata, and other marine forms. M. E. KRAHL, A. K. KELTCH, C. P. WALTERS AND G. H. A. CLOWES.

The following values for hexokinase activity in homogenates of eggs and embryos were obtained (micrograms glucose used at 20° C. per hour per mg. protein); unfertilized eggs, 67; eggs fertilized one hour, 72; 24-hour plutei, 155; 48-hour plutei, 226. The hexokinase activity of the unfertilized egg homogenates was virtually all recovered in the supernatant fraction when the homogenate was centrifuged at 20,000 g. for 30 minutes. Relative rates of phosphorylation of various sugars by the supernatant fraction when saturated with substrate were, approximately: glucose, 1.0; mannose, 1.2; fructose, 1.8; 2-desoxyglucose, 2.0; glucosamine, 0.6; galactose, 0.0; fructose-6-phosphate, 0.2; glucose-6-phosphate, 0.0. The rate in presence of saturating concentrations of two substrates was in no instance significantly greater than the highest rate for one substrate of the pair; thus, there is no evidence for more than one hexokinase. The concentrations for half maximal hexokinase activity with various substrates were, approximately: glucose, 0.00003 *M;* fructose, 0.00075; mannose, 0.00007; 2-desoxyglucose, 0.00025. The *Arbacia* hexokinase differs from that of other animal tissues with respect to the concentrations of various sugars required for saturation, as well as in the relative maximum rates. Hexokinase activity was found in eggs of another echinoderm, *Echinarachnius*, but none in eggs of the annelid, *Chaetopterus*, or of the mollusc, *Mactra*. The isomerase activity in the supernate fraction from *Arbacia* is sufficient to convert glucose-6-phosphate to fructose-6-phosphate at about 20 times the rate at which the hexokinase present can form glucose-6-phosphate from glucose. No myokinase was present in the supernatant fraction.

Cholinesterase and ciliary activity in the gill of Mytilus. SAMUEL P. MARONEY, JR., AND R. R. RONKIN.[1]

Recently Seaman (1951) and Kordik (1952) have indicated a relationship between cholinesterase and ciliary activity. In the present investigation, cholinesterase activity of ctenidial (= gill) homogenates of *Mytilus edulis* L. was determined by the standard Warburg procedure for carbon dioxide production. Although the rate of spontaneous breakdown of acetylcholine was not large enough to be detected under our experimental conditions, the rate of hydrolysis in the presence of homogenate increased to $Q_{ACh} = 3.47 \pm 0.20\lambda$ CO_2 per hour per mg. (dry). The enzymic activity was completely inhibited by eserine at concentrations of 10^{-5} *M* or greater.

The rate of movement of gill fragments along the bottom of a Petri dish of buffered (0.03 *M* glycine, pH = 8.0) sea water was used as a measure of the rate of ciliary movement. The effect of eserine on ciliary activity was tested by measuring the rate of movement before and after the addition of the inhibitor. The effect of eserine was two-fold: ciliary activity was reversibly inhibited at 10^{-3} *M* eserine and was reversibly enhanced at 10^{-4} *M* and 10^{-5} *M* eserine. The concentration of eserine for maximal enhancement of ciliary activity, 10^{-4} *M*, did not have a measurable effect on oxygen consumption (Warburg) of gill fragments.

If one assumes that the inhibitor is effective in the interior of the intact cell at the same concentration as that which obtains outside, the complete inhibition of cholinesterase activity plus the apparent failure to inhibit oxygen consumption of fragments with enhanced ciliary movement, suggest that there may be a relationship between this enzyme and ciliary activity in *Mytilus.*

The electrochemical potential difference of the chloride ion in the giant squid axon-sea water system. ALEXANDER MAURO.

It has been postulated many times in the literature on axon physiology that the chloride ion, among others, might be in thermodynamic equilibrium with the extracellular fluid. This condition can be determined unambiguously by invoking the electrometric property of a pair of identical reversible electrodes, namely, that the electrostatic potential developed at the metallic wires of the electrode system is proportional to the electrochemical potential difference existing between the electrode coordinates. Thus if a condition of thermodynamic equilibrium were to exist between two phases the electrostatic potential developed by the electrode pair should be zero.

An attempt was made to construct a reversible silver-silver chloride electrode by cementing a fine silver wire (15 micra) in a glass capillary. The ability of the electrode to behave as a "chloride electrode" was investigated by observing the potential developed by the electrode versus a calomel half-cell in solutions of varying chloride concentrations. Moreover, the electrode maintained a stable "zero" potential of fractions of a millivolt for many days versus a macroelectrode in a solution of given chloride concentration.

Although this communication is to be regarded as a mere preliminary note, measurements on twelve axons in sea water seem to indicate that the chloride ion is not in thermodynamic equilibrium. This conclusion is warranted by the fact that the "resting potential" as developed between the internal and external silver-silver chloride electrodes was of the order of thirty-five millivolts (internal negative).

Effect of some antibiotics on the cleavage of Arbacia eggs. YOSHIAKI MIURA.

During the course of an investigation to find new antibiotics which will be effective in curing ascites tumor of rats, Umezawa *et al.* recently have found two effective substances from *Streptomyces thioluteus,* designated as mitomycin and 4-A-1. The present investigation was undertaken to test the effects of these and other antibiotics on the cleavage of Arbacia eggs.

[1] Supported by the Faculty Research Committee of the University of Delaware, and by a contract between the University and the Office of Naval Research, Department of the Navy (NR 160-015).

The concentration of test substances which inhibit or retard cleavage of Arbacia eggs are, respectively: Aureothricin (*Streptomyces albus*), 10 mg./L and 1 mg./L; 4-A-1, 100 mg./L and 10 mg./L; Achromycin (*Streptomyces alboniger*), 1000 mg./L and 100 mg./L; Mitomycin, 100 mg./L and 10 mg./L; colchicine, 500 mg./L and 20 mg./L; podophyllotoxin, 10 mg./L and 5 mg./L; amino-chlor dibenzofurane (a synthetic usnic acid derivative), 10 mg./L and 1 mg./L. Saturated solutions of Actinomycin-A (*Actinomyces flavus*) and Viscosin (*Pseudomonas viscosa*), even at 100 mg./L, only slightly retarded the cleavage rate. Aureomycin shows complete and irreversible inhibition at 1000 mg./L but no inhibition or even retardation at 100 mg./L. Amino-chlor benzofurane showed irreversible inhibition at 10 mg./L. The actions of the other antibiotics except mitomycin are reversible when the eggs are removed to fresh sea water after one hour immersion with inhibitory solution.

Mitomycin displays a distinctive kind of action on the eggs. The total inhibition of the cleavage occurred at the concentration of 100 mg./L. At 10 mg./L, the first cleavage was retarded but morphologically normal until near the end of the furrowing process. Then cytoplasmic blisters appeared in a contrast group on both blastomeres, at the outer edge of the furrow. As a rule these eggs did not divide again, although eggs which did not bleb underwent a second cleavage. These images are entirely different from those which one can observe with colchicine or podophyllotoxin.

Qualitative analysis of acid-soluble, purine and pyrimidine bases in developing Echinarachnius eggs. YOSHIAKI MIURA AND ROBERT J. NEFF.

Aliquots (containing $3\text{–}4 \times 10^5$ individuals) of *Echinarachnius* unfertilized eggs, or first cleavage, early blastula, early gastrula, and prism embryos were examined. Samples were packed centrifugally, chilled in a cracked-ice bath, homogenized in 1.5 ml. of cold 1% perchloric acid, and cold-extracted twenty minutes. Residues were removed by centrifuging 10 minutes at 10,000 rpm in a Serval high speed centrifuge and the supernatants used for analysis by chromatography on Whatman number one paper at room temperature. The three following solvent mixtures were used in the chromatography: (1) n-butanol, 3 vols.; ethanol, 2 vols.; 5 N HCl, 2 vols.; (2) n-butanol, 86 vols.; water, 14 vols.; (3) solvent (2), 95 vols.; 28% NH_4OH, 5 vols. Synthetic purine and pyrimidine bases were used as controls in the chromatograms. Spots of known and unknown samples were detected with an ultraviolet lamp and Rf values calculated. Spots were eluted with 0.1 N HCl and further characterized by their ultraviolet absorption spectra. The small amount of echinochrome pigments in *Echinarachinus* eggs did not interfere with the analyses.

Appreciable amounts of guanine and adenine were found in all stages examined. Hypoxanthine was present up through the gastrula stage, but was not detectable in the prism stage. Xanthine appeared only in the prism stage. There was also an unidentified base having the same Rf value as uracil present in unfertilized eggs and embryos up through the gastrula stage. Like hypoxanthine, this material disappeared after gastrulation. That this material was not uracil was indicated by its absorption maximum and minimum in 0.1 N HCl. They were 268 and 245 millimicrons, respectively, for the unknown material, as compared to 260 and 230 millimicrons for uracil extracted and read under the same conditions.

Protamin in an extract of the sperm of Nereis limbata. W. J. V. OSTERHOUT.

Nereis limbata was collected from the Marine Biological Laboratory wharf on the Eel Pond before June fifteenth. Soon after collecting, the "dry sperm" was obtained as a soft white mass, avoiding contamination as much as possible. A standard solution of salmon protamin was used to determine the adequacy of the method employed. The suspension of live sperm in sea water at various concentrations showed absence of protamin when the suspension was centrifuged and the supernatant liquid tested.

When sufficient sperm was suspended in sea water and heated at 55 degrees centigrade for ten minutes, allowed to stand for several hours after which it was centrifuged and the supernatant liquid was removed and tested, the presence of protamin was clearly indicated. In the absence of the reagent the test was negative. This indicated that protamin was extracted from the dead sperm by heating. An extract of the dead sperm capable of activating the egg was

too dilute to show a test for protamin. A similar result was obtained with *Nereis limbata* collected from Vineyard Sound in August except that the season was too late for the activation experiment. Salmon protamin in sea water at a concentration that gave a positive protamin test produced no persistent foam when bubbles of air were passed through it before and after heating for ten minutes at 55 degrees centigrade. This indicated that the salmon protamin was not surface active.

Surface active material obtained from Nereis limbata. W. J. V. OSTERHOUT.

Nereis limbata was collected from Vineyard Sound. Soon after collecting, the "dry sperm" was obtained as a soft white mass, avoiding contamination as much as possible. The sample consisted of one part of "dry sperm" and nine parts of filtered sea water. This was centrifuged. The supernatant liquid was removed and bubbles of air passed through it at room temperature. Persistent foam was produced which indicated the presence of surface active material. The same test for surface active material was made in subsequent experiments. The supernatant liquid was replaced by an equal volume of sea water which was mixed with the sperm and centrifuged. This supernatant liquid was removed and the test showed little or no surface active material. This indicated that it had been removed by one washing. On heating this supernatant liquid for ten minutes at 55 degrees centigrade and testing, no surface active material was detected. This indicated that no surface active material was produced by heating. The supernatant liquid was replaced by an equal volume of sea water which was mixed with sperm. This was heated for ten minutes at 55 degrees centigrade and centrifuged. The supernatant liquid was removed and tested. This showed the presence of surface active material. This indicated that surface active material was extracted from the dead sperm on heating. A similar result was obtained when the "dry sperm," appearing as a soft pink mass, was contaminated with blood and possibly other material from the body.

The pH values of the supernatant liquids above described were a few tenths of a pH lower than that of the sea water but on heating them the pH values became about the same as that of the sea water indicating the presence of volatile material, possibly carbon dioxide. These differences in pH values did not affect the surface activity.

Release of an anticoagulant from irradiated Spisula eggs.[1] PETER RIESER AND ALVIN M. KAYE.

In the hope of obtaining information concerning the effect of roentgen rays on the colloidal properties of protoplasm and in the hope also of interpreting the heparinemia occurring in the blood of mammals as a result of whole body irradiation, an attempt was made to demonstrate the release of heparin or heparin-like substances from isolated irradiated cells. The eggs of the clam Spisula contain a powerful anticoagulant (Thomas, 1951). These eggs were suspended in sea water and washed five times to remove the jelly surrounding them. One-half of the suspension was then exposed to 200,000 r, the other half used as a control. Both lots of eggs were then centrifuged, and the supernate incubated with crystalline trypsin, which was subsequently destroyed by boiling. The resultant solutions were centrifuged and the supernate assayed for anticoagulant activity with sheep plasma, using purified thrombin as an activator. The solution from irradiated eggs produced delays of clotting of as much as 48 hours, whereas the control solution from non-irradiated eggs allowed clotting to occur within a few minutes. Addition of toluidine blue to the solutions from the irradiated eggs produced strongly metachromatic precipitates, whereas similar additions to the control solutions did not. Removal of this precipitate from the solutions destroyed their anticoagulant activity. Preliminary studies on the eggs of the sea urchin Arbacia and the worms Chaetopterus and Arenicola showed no effect of radiation on the release of anticoagulants. This may in part be due to the fact that Spisula eggs produce more anticoagulant; in part also to the fact that the Spisula anticoagulant has a more powerful effect on vertebrate blood (*cf.* Thomas, 1951). Similar investigations are planned for various types of vertebrate cells.

[1] This investigation was supported by a research grant from the U. S. Atomic Energy Commission, administered by L. V. Heilbrunn.

Surface activity of hypobranchial mucus of Busycon: possible nutritive function of mucous substances. R. R. RONKIN.[1]

Fresh hypobranchial mucus of the large prosobranch gastropod, *Busycon canaliculatum* (L.), lowers the interfacial tension between air and the sea water in which it is dissolved. The lowest value obtained, 47.5 dynes cm.$^{-1}$, was 64% of that obtained with pure sea water (du Noüy method; t = 22° to 25° C.). Graphical solutions of the Gibbs adsorption equation indicate that the greatest excess of solute concentrated in the surface phase occurred when the mucus was diluted about 10 times with sea water. Estimates of solute concentration suggest that under these conditions the concentration (w/v) of mucus in the sea water was between 0.01% and 0.05%. Although it is certain that different animals produce mucus with widely diverse behavior in solution, it is possible that the surface activity of some types of mucus solutions is important in feeding, in that it improves the wettability of food particles.

The effect of x-rays on the ability of marine egg protoplasm to undergo two colloidal reactions. ALBURT M. ROSENBERG.[2]

X-irradiation may not produce a measurable colloidal change in the unfertilized egg but may alter the ability to undergo such required changes (Wilson, 1950). In two situations involving colloidal change, the behavior of irradiated eggs was compared with that of control eggs.

Cerebratulus lacteus eggs in the metaphase of the first maturation division were treated with sea water made hypertonic with calcium chloride (Yatsu, 1905) to form numerous asters in the cytoplasm. The cytasters were observed in fixed whole-mount preparations under phase microscope and in the living cells with dark field illumination. Data gathered a half hour after final treatment showed eggs irradiated with 100,000 r, a dose which significantly reduced the size of the metaphase figure, have many very small vesicles (cytasters in the fixed) while the non-irradiated had fewer but larger vesicles. This difference is caused by the many vesicles originally present in the control coming together as they approach the center of the cell. The viscosity of the eggs with cytasters was considerably higher than that of untreated eggs.

Arbacia punctulata eggs put into hypotonic sea water usually break at one point. Several seconds later these eggs appear suddenly pink under dark field. The time from breaking at the cell membrane to pigment granule lysis was slightly longer for eggs previously given 20,000 r than for the control. However, experimental reproducibility was not entirely satisfactory.

Future research should include determinations of viscosity in irradiated and control eggs subsequent to treatment with agents which increase and which decrease the viscosity of the protoplasm. Perhaps the action of x-rays may best be understood not as an alteration of the colloid itself but rather as an effect on the agents which bring about colloidal changes in the living cell.

Persistent diurnal rhythms of O_2-consumption in the periwinkle, Littorina littorea, and the oyster drill, Urosalpinx cinereus. MURIEL I. SANDEEN, GROVER C. STEPHENS AND FRANK A. BROWN, JR.

The O_2-consumption of the common periwinkle, *Littorina littorea*, was studied using an automatic, continuously-recording respirometer. A group of 6 or 8 snails was placed in each of four respirometers and their respiratory rate followed for about a 48-hour period. These were then replaced by freshly collected animals. Throughout a period of 26 days a total of about 400 snails was used. It was found that the average value of O_2-consumption in all experiments was about 9 ml./kg./hr. Large variations in rate of O_2-consumption were observed in each daily period. These variations in oxygen consumed occurred in such a manner as to

[1] Aided by a grant from the Faculty Research Committee of the University of Delaware, and by a contract between the University and the Office of Naval Research, Department of the Navy (NR 160-015).

[2] Predoctoral Fellow, Atomic Energy Commission.

suggest the existence of a diurnal pattern. For two 15-day periods the average O_2-consumption per snail per hour was tabulated and an average value obtained for each hour of a daily period. In this way any possible persistent tidal variations were eliminated. The two resulting daily curves of O_2-consumption clearly demonstrated the existence of a diurnal rhythm in metabolism. The metabolic rate is lowest about 2 to 3 A.M., increases rapidly to a maximum value about 8 to 9 A.M., and declines steadily to a second minimum value about 2 P.M. Thereafter, there is a gradual increase to a second maximum at approximately 10 P.M. The values through the daily cycle ranged in magnitude from a minimum of about 5 to a maximum of about 13 ml./kg./hr. A comparable investigation of the snail Urosalpinx revealed a similar persistent diurnal rhythm in metabolism but with times of maxima and minima slightly different.

Osmotic and other studies of the Spisula egg. VICTOR SCHECHTER.

The osmotic and tensile properties of the Spisula egg were studied by the means of allowing the cells to come into equilibrium with diluted sea water of graded concentrations. Preliminary tabulation of results indicates a linear relationship between dilution of the medium and the amount of swelling of the egg, in the range where no damage to the egg surface occurs. Data were also obtained on the osmotic properties of the nucleus, which enlarges measurably when the egg is placed in a hypotonic medium. Characteristic indentation of the cortex, which is a feature of aging of the unfertilized Spisula egg cell, can be induced with vitamin K. This effect does not occur in the absence of calcium. The effect of vitamin K upon the osmotic properties of the egg membrane is under investigation.

Discontinuous carbon dioxide output by diapausing pupae of the giant silkworm, Platysamia cecropia. HOWARD A. SCHNEIDERMAN AND CARROLL M. WILLIAMS.

Using the "direct" Warburg manometric method, it has been shown that diapausing pupae of the Cecropia silkworm release carbon dioxide in discrete bursts, confirming the diaferometric CO_2 measurements of Punt on other species. Oxygen consumption, by contrast, exhibits no such discontinuities. In a typical pupa weighing five grams and having a steady oxygen uptake of 70 $mm.^3$/hour at 25° C., the sudden release of CO_2 occurred regularly once every eight hours. At lower temperatures the bursts were less frequent; at 10° C. they occurred about once every three days. Each burst lasted about 30 minutes and accounted for 95% of the total CO_2 output. The remaining 5% was given off continuously. The over-all R. Q. measured over a two-day period was 0.78. That virtually all the gas exchange takes place via the spiracles may be shown by occluding them with wax.

The frequency of CO_2 bursts increases with increasing metabolic rate until, when oxygen uptake exceeds 160 $mm.^3$/gram live weight/hour, the bursts disappear and CO_2 evolution becomes continuous. Such animals when placed at 10° C. show a reduction in metabolism, and CO_2 output again becomes discontinuous.

A larger percentage of the total CO_2 was given off continuously at high burst frequencies than at low burst frequencies; when bursts occurred at one-hour intervals, more than half the CO_2 was released continuously in the inter-burst period.

Exposure to 6% O_2, while not affecting O_2 uptake, resulted in the disappearance of CO_2 bursts. Exposure to 10% CO_2 also eliminated the discontinuous release of CO_2. Isolated anterior halves of pupae and isolated pupal abdomens both gave off CO_2 bursts.

An explanation of the burst phenomenon consistent with the above observations will be offered subsequently.

A rare poly-anomalous embryo. D. R. SHANKLIN.

In with several hundred normal *Fundulus heteroclitus* embryos, a four-day specimen was found anomalous. It was followed to eight days, dechorionated, fixed in Bouin's, serially sectioned, and stained with BPB. These external anomalies were apparent: monophthalmia with two lenses; no oral depression; a bulbous mass of red tissue in place of the right pectoral fin;

similar tissue adjacent to the tail fin; a single hypertrophied urinary vesicle, without its midline constricture. Proceeding caudad, an inspection of the sections shows the following anomalies: reduced telencephalonic evaginations, which are displaced dorsocaudad to the monophthalmia, which opens ventrally; diencephalonic fusion with reduction of the third ventricle to a cord of ependymal cells; absent oral cavity; the lining cells of the pharynx contain a dense granular metachromatic material which may be the hatching enzyme precursor; the mesencephalonic aqueduct is typical for a brief spell, then vanishes; the fourth ventricle is enlarged, and the alar plates are absent; a discrete cleft occurs between ventral basal plate and notochordal tissue, concurrent with the auditory vesicle, which is enlarged; the pectoral fin dismorph, an aberrant splenic primordium, and the greatest accumulation of the metachromatic material occur at the decresence of the myelencephalon; median pronephri are joined by lateral pronephri, which proceed through many sections and fuse caudad to the convolutions, the left pronephros crossing the midline and continuing thus to the vesicle; the swim bladder is displaced caudad and to the right; here the sclerotomic mesoderm is replaced by transversely oriented striate muscle, the band intermittently passing to fuse with the longitudinal bundles about the spinal column; the aberrant tail tissue is also splenic.

The development of isolated blastomeres of Fundulus heteroclitus.[1] JOHN R. SHAVER.

Previous analyses of the developmental potencies of the early blastomeres of *Fundulus* have been made by destroying cells and observing the development of surviving blastomeres. The present experiments parallel those of Tung on *Carassius*, in that both fragments resulting from the separation of the 2- or 4-cell stages have been raised. After dechorionation in sterile medium, the blastoderm was separated along the first cleavage furrow continuing through the yolk-sac. From one to two thirds of the yolk was lost in the operation, and from over 200 such isolations only 50 pairs of fragments successfully healed and cleaved. Cleavage followed an essentially normal pattern in most cases, and a majority of fragments proceeded to gastrulate, either partially or completely, apparently depending on the amount of yolk proportional to the blastoderm. Controls consisted of 2-cell stages dechorionated but otherwise unoperated. A very small percentage of the healed isolates proceeded beyond gastrulation and in no case so far have embryos been obtained from both members of the pair or fragments. One case of a normal but small embryo has been gotten from one of the first two cells; the other member of this pair did not form an embryonic axis. Another small but normal embryo developed from two of the first four cells. In many cases embryonation proceeded in one or both fragments to some extent but failed. Much reduced embryos, poorly differentiated, resulted in about 10% of cases. Most numerous were fragments in which vesicular forms, with or without "tail-like" structures protruding from the blastoderm, developed; in some of these blood and/or pigment cells differentiated on the yolk-sac.

Surface active properties of an extract of the sperm of Nereis limbata. THEODORE SHEDLOVSKY AND W. J. V. OSTERHOUT.

It has been stated by one of us (this journal, Vol. 103, No. 2, 305–306, October, 1952) that an extract of dead sperm which can activate the egg contains surface active material, and that purified Duponol in sea water also was found to be activating.

Extract, S, of dead sperm in sea water did not alter the osmotic pressure of sea water within one per cent at the concentration capable of activating the egg.

The surface tension of various dilutions of S showed a rapid decline from zero concentration reaching an essentially constant value of about 2/3 the surface tension of sea water above ten-fold dilution of S. A similar surface tension-concentration curve was obtained with purified Duponol which reached a surface tension of about 1/2 that of sea water above 0.002% concentration. This corresponds to the critical concentration above which micelles are formed. Studies of surface films revealed that S forms high surface viscosity films, with slow drainage

[1] Supported by funds from U. S. Public Health Service.

up to 55° C. above which no stable films could be made. In contrast, purified Duponol films drained rapidly even at 0° C., showing relatively low surface viscosity. Such behavior is characteristic of foam-producing substances free of slightly soluble alcohols or fatty acids. When such are present slow draining films with high surface viscosities are formed, but these can be transformed into rapidly draining films by raising the temperature. It appears that the surface active properties of S may be due to one or more foam-producing substances which also contain a slightly soluble material such as an acid or alcohol. Since surface tension (liquid-gas) behavior is not necessarily paralleled in interfacial systems it would be of interest to continue these studies with observations on sperm extract-oil interfaces.

The role of the vitelline membrane in the fragmentation of the Arbacia egg by centrifugal force. SISTER FRANCIS SOLANO AND DANIEL MAZIA.

The centrifugal force required to break a cell into two fragments has been used in the study of the physical properties of the cell surface. It has been recognized that the measurements apply to a complex of surface layers. In the sea urchin egg, one of these is the vitelline membrane which is transformed into the fertilization membrane on activation. This membrane may be removed by means of proteolytic enzymes, after which activation and development may occur, but no fertilization membrane is seen.

A mixture containing one mg. of crystalline trypsin and one mg. of crystalline chymotrypsin in sea water was effective in removing the vitelline membrane of *Arbacia* eggs. Such eggs fertilized and developed normally. Control eggs and eggs from which the membranes had been digested were centrifuged simultaneously. None of the controls fragmented at the limiting force at which 100% of the membraneless eggs fragmented. A force at least 25% greater was required for fragmentation of the controls. Therefore, the vitelline membrane contributes significantly to the resistance of the egg to fragmentation, and is an important component in what has been described as the tension at the surface of the egg.

It had been found earlier by E. S. G. Barron that uranium ions in low concentration greatly increase the resistance of eggs to fragmentation. We have confirmed this, employing 5×10^{-4} *M* uranyl nitrate, and obtain the same results in the presence and absence of the vitelline membrane.

Electric current distribution in a multicellular system as determined by the pattern of biological events resulting from exposure of developing Rana pipiens eggs to externally applied elliptically polarized currents. RICHARD N. STEARNS.

Developing *Rana pipiens* eggs were exposed to elliptically polarized electric currents, the frequency of rotation of the current vector varying between zero and 6000 cycles per second. This type of current pattern was used to eliminate membrane polarization effects. The eggs were in the field for ten minutes at different stages of development, and visible damage was assayed immediately under a dissecting microscope. Changes in developmental pattern were followed in whole embryos and in histological sections. The distribution of events arising when eggs are treated between 0.25 and 5.0 times threshold at different developmental stages are as follows: 1) The damage is mainly restricted to the interfaces between cells. 2) This damage pattern does not result from a corresponding sensitivity pattern since the per cent of eggs which show visible damage restricted solely to the interfaces at the 2-cell, 4-cell and 8-cell stages, are 10%, 50%, and 100%, respectively, with the voltage range employed in these experiments. 3) The per cent mortality decreases when eggs are treated with greater cell numbers. 4) The per cent of eggs which cleave but do not differentiate is low when treated before first cleavage, rises sharply at the 2-cell stage and declines with a slope approximately like that of the mortality curve. 5) The per cent of eggs where only the vegetal half is destroyed is similarly related to cell number. (6) Alteration in developmental pattern is low before first cleavage, rises sharply at the 2-cell stage, declines with increased cleavage number, and rises again at approximately mid-cleavage and again decreases between small yolk-plug and tail-bud stages. These results indicate that the biologically effective current is an extra-

cellular current (an ionic current as indicated by the threshold-frequency curves) such that cell membranes must be damaged before the interior of the egg is affected, when the cell number is greater than one.

Alteration in developmental pattern arising from cell membrane damage caused by elliptically polarized electric currents in Rana pipiens eggs. · RICHARD N. STEARNS.

The eggs described in this abstract were ones treated in the manner described in the previous abstract. Neurulae which had prior electrical treatment stop visible differentiation at the tail-bud stages for 24–56 hours at 18 degrees after which time 85% of the stopped embryos become normal tadpoles. The irreversibly inhibited embryos show non-specific damage in the three germ layer derivatives, or differentiation may be stopped entirely. If the voltage gradient range is increased to 6 times threshold, the per cent of irreversibly inhibited embryos increases to 50%.

Embryos may be stopped in gastrulation by treatment as early as the 2-cell stage and as late as the small yolk plug-gastrulae stages. After a period where no obvious mass cell movements are observed, development proceeds without gastrulation, the embryos oriented with reversed dorso-ventral axes. The embryos are internally twinned, the two systems forming directly across the yolk-plug and a swimming tadpole will result with a regionally well-differentiated nervous system. Thus the regional differentiation has occurred without gastrulation.

Animal caps, which are produced by destroying the vegetal halves of developing eggs and subsequently left in contact with the killed material, will differentiate ectodermal, mesodermal, and endodermal derivatives, development proceeding in the same way as the stopped gastrulae. In eight cases, swimming tadpoles resulted with good regional differentiation. Two caps which healed over so that they did not remain in contact with the killed area showed no differentiation. Thus it is suggested that the gross geometry is important not only for histogenesis but also organogenesis, and the killed material may also play a purely chemical role in the differentiation and organization of the animal cap.

Membrane potential changes in fibers of the frog sartorius muscle during sodium extrusion and potassium accumulation. WILLIAM K. STEPHENSON.

When nerve or muscle is in a steady-state condition, the membrane potential may be expressed by the familiar Nernst equation. In accord with this expression, when K^+ is added to the bathing solution the potential decreases; however, if K^+ is injected into the fiber (Grundfest and others), the membrane potential does not increase in accord with theory. This report deals with membrane potential changes taking place in muscle fibers greatly depleted of K^+ during recovery from this condition.

Frog sartorii were dissected and soaked for 24 hours in K-free saline (0.12 *M* NaCl + 0.005 *M* Na–PO_4 buffer, pH = 7.2) at 2° to 6° C., during which time muscle K^+ content fell to 30.3 ± 4.6[1] mM./Kg. fiber weight (mM.) and Na^+ increased to 98.2 ± 5.3 mM. These muscles were then transferred to high-K recovery saline (0.11 *M* NaCl + 0.01 *M* KCl + buffer) at room temperature for 55 minutes, where their K^+ content rose to 72.2 ± 3.1 mM. and Na^+ dropped to 59.2 ± 5.4 mM. Membrane potentials were measured at the beginning and end of the recovery process by the Ling and Gerard glass microelectrode technique, and readings of 43.9 ± 1.6 mV. and 46.1 ± 1.5 mV., respectively, were obtained. Control muscles suffered drops of membrane potential during recovery as follows: soaked and recovered in K-free saline, 46.3 ± 1.9 mV. to 41.3 ± 1.9 mV.; and soaked and recovered in high-K saline, 52.3 ± 0.9 mV. to 48.1 ± 0.9 mV.

Calculations of the membrane potentials expected on the basis of the Nernst equation give values of 28 mV. before recovery and 50 mV. after recovery, yet the recorded potential at the onset of recovery was markedly higher than theoretical and that at the termination,

[1] Standard Error of the mean.

somewhat lower. These results indicate that the membrane potential is not necessarily dependent upon the K^+ distribution, and that the Nernst equation can not, therefore, be applied in cases of rapid ion shifts.

(This work was aided in part by Contract Nonr–24900, Office of Naval Research, administrated by H. B. Steinbach.)

A note on specific pigment in the lateral line receptor cell. GEORGE C. THRASHER.[1]

Recently Allison (1953) has suggested that sight, smell and hearing all require the presence of pigment for the proper performance of their receptor cells. This investigation is concerned with the pigment of still another group of receptors, those of the lateral line system. Bedell (1939) called attention to this pigment in the hair cells of lateral line organs of tadpoles and salamander larvae. In freshly collected specimens it appears as clumps of bright orange-yellow granules. These survive sectioning and routine staining. Two questions immediately arise about this material: (1) Is it necessary for the proper performance of the receptor cells? (2) What is its chemical nature? If the answer to the first question is yes, then it would seem that an identical or at least very similar pigment would be required by other forms possessing a lateral line system, particularly fishes. Whole and teased fresh mounts and stained and unstained frozen sections of lateral line organs of dogfish, skate, catfish and others were examined for granules resembling those apparent in similarly treated organs of tadpoles and salamanders. None were observed. If this finding is substantiated by further investigation it would seem to indicate that the pigment of these receptors is of no functional significance as far as the reception of impulses or the transmission of stimuli is concerned. The initial color of the known granules and the color changes which they undergo as they degenerate under laboratory conditions are reminiscent of rhodopsin (visual purple) changes. Histochemical tests, however, demonstrate that the granules are not carotene or carotenoid.

Nucleic acid turnover rates in Arbacia eggs and sperm. CLAUDE VILLEE, DOROTHY VILLEE AND RUTH LAPLACE.

A solution containing 25 microcuries of P^{32} in 0.2 ml. of sea water was injected into the coelomic cavity of adult *Arbacia*. The urchins were then kept in running sea water for 24 hours, after which eggs and sperm were collected either by KCl injection or by dissection. The eggs and sperm were washed in sea water, and then an aliquot of the eggs was fertilized by sperm from uninjected males and allowed to develop for eight hours. The sperm, unfertilized eggs and fertilized eggs (swimming gastrulae) were killed by the addition of trichloracetic acid, homogenized, and fractionated by the method of Schmidt and Thannhauser. Phosphorus was determined by the method of Fiske and Subbarow and radioactivity by a Geiger dip counter. The experiments showed that no P^{32} was incorporated into the desoxyribonucleic acid (DNA) of the sperm *in vivo*, but some was incorporated into the sperm ribonucleic acid (RNA) fraction. Radiophosphorus was incorporated into the RNA of unfertilized eggs *in vivo* slightly faster than into the DNA. Upon fertilization and during development in P^{32}-free sea water, the amount and specific activity of the DNA fraction increased two- to four-fold, whereas the amount and specific activity of the RNA fraction increased only slightly. The DNA of mature sperm, in contrast to that of unfertilized eggs, does not incorporate phosphorus *in vivo*. A comparison of the specific activities suggests that the phosphorus of the DNA synthesized during early development is derived from the acid-soluble phosphorus fraction.

Relationship between time of day and inhibiting influence of low temperature on the diurnal chromatophore rhythm of Uca. H. MARGUERITE WEBB, MIRIAM F. BENNETT, ROBERT C. GRAVES AND GROVER C. STEPHENS.

Exposure of *Uca pugnax* for short periods to a temperature of 5° C. was found to inhibit the diurnal rhythm as observed in the black chromatophores. In one experiment groups of

[1] This work was supported by a Massengill Fellowship.

50 animals were placed in a darkroom at 7 P.M. and remained in the dark for the duration of the experiment. Each group was exposed to a temperature of 5° C. (in a refrigerator) in darkness for a single 6-hour period in a different quarter of the day. Following this treatment the average stage of the chromatophores was determined at hourly intervals from noon until the animals reached the night phase on five successive days. In all cases a shift in the phases of the diurnal rhythm was observed but in no case did the extent of the shift equal the chilling period. When the extent of inhibition was calculated by comparison with a control group, it was seen to vary in a regular manner with the time of day at which the animals were chilled. The times of day and amounts of inhibition obtained were as follows: 12 P.M. to 6 A.M., 72%; 6 A.M. to 12 noon, 56%; 12 noon to 6 P.M., 63%; and 6 P.M. to 12 P.M., 78%. When two-hour chilling periods were used, 79% inhibition was found when chilling occurred from 8 P.M. to 10 P.M., and only 24% with chilling from 10 A.M. to 12 noon.

Studies of the effects of x-radiation upon Paramecium: variations among and within species.[1] RALPH WICHTERMAN.

Clones of seven species of *Paramecium* were irradiated in gradually increased dosages up to 500 kiloroentgen using Nylon syringes as irradiation chambers. Specimens were cultivated in lettuce medium with *Aerobacter aerogenes* as the bacterial food and irradiated at the growth phase. Four syringes, free of air, were irradiated simultaneously. Each experiment involved 800 specimens (200 per syringe in 2 cc. culture fluid). After each x-ray dosage, countable numbers—commonly 10—were expressed from the syringes, placed in spot plates in moist chambers and observations continued periodically to 48 hours. Variations in x-ray susceptibility exist not only among species but within them. Under fairly uniform cultural and x-ray conditions, if percentage of survivors is computed against x-ray dosage and attempts made to obtain an LD 50 for 24-hour periods, it is observed that the species may be arranged in sensitivity to x-rays with *P. calkinsi* the most resistant (LD 50: 400 kr.) and *P. trichium* the most sensitive (LD 50: 170 kr.) as follows: *P. calkinsi, P. multimicronucleatum, P. bursaria, P. caudatum, P. aurelia, P. polycaryum, P. trichium.*

X-ray susceptibility is dependent not only upon the concentration of bacteria and dissolved oxygen in the medium with paramecia, the chemical constitution of the culture fluid and changes brought upon it as a result of ionizing radiations (such as production of hydrogen peroxide) but also the phases and stages of growth of individual paramecia. When the results of many experiments are plotted to yield per cent survival curves they frequently are not sigmoid. Instead the slope of the curve is so steep approaching lethality as to be almost vertical. However before the steep drop, commonly two peaks occur. These peaks or variations from the typical curve are greater than the biological variation expected and indicate definite sensitivity levels and differences in specimens being irradiated. These sensitivity thresholds, plus conditions of irradiation and the fact that many immobilized specimens may remain seemingly dead for hours thru the 24-hour period only to recover later from irradiation effects, may explain the difficulty in obtaining consistently uniform LD 50 values.

Pharmacological studies on marine invertebrates. CLARE M. WILBER AND SR. ELIZABETH SETON.

This is a report of progress on a study of the effects of octamethyl pyrophosphoramide (OMPA), hexamethonium (C6), and decamethonium (C10). Changes in the glucose content of the body fluids from *Amphitrite ornata* and *Phascolosoma gouldii* and in the blood from *Libinia emarginata* were recorded. In general, treatment with any of these agents, by immersion or by injection, results in a drop in the glucose of the body fluid from the animals studied. For example, in *Phascolosoma* immersed in C6 (one g. per 1200 ml. sea water) the glucose of the body fluid decreased from an average of 19 to 16 mg. per 100 ml.; in the same concentration of C10 the decrease was from 24 to 20 mg. per 100 ml.; after immersion in OMPA the glucose decreased from 14.2 to 8.5 mg. per 100 ml. body fluid. After injection

[1] Aided by a grant from the Committee on Research, Temple University and performed under Contract NR 135-233, Office of Naval Research.

of 5 mg. of C10 into *Libinia* a similar decrease in blood sugar resulted: from about 5 to 1 mg. per 100 ml. Injections of C6 also brought about a decrease in blood sugar in the spider crab. A similar situation obtains in *Amphitrite: e.g.* after C6 injection a decrease in glucose from 29 to 23 mg. per 100 ml. body fluid; after immersion in OMPA like decreases are found. Since C10 imitates acetylcholine (ACh) in its action and OMPA inhibits cholinesterase, one would anticipate that their effects would be similar. On the other hand C6 antagonizes acetylcholine. The excitation of the animals induced by accumulation of acetylcholine (OMPA) or by the action of an ACh-like material (C10) may deplete the glucose in the body fluids faster than it can be replaced from storage tissues. A study of the tissue glycogen of these animals, treated as in the present work, is planned.

The action of cyanide on the protoplasmic colloid.[1] WALTER L. WILSON AND L. V. HEILBRUNN.

As has been long known, dilute solutions of potassium cyanide prevent the division of marine eggs. The effect has generally been attributed to the fact that cyanide inhibits the activity of some of the respiratory enzymes. But the division of a cell is a mechanical process dependent on forces developed in the protoplasmic colloid. Hence it is of interest to know how the colloidal changes that occur during the division of a cell are affected by cyanide. In the egg of the worm Chaetopterus, the mitotic gelation is prevented by solutions of cyanide —that is to say, the protoplasm in the interior of the egg stays fluid instead of clotting to form a mitotic spindle. Similarly, in the egg of the clam *Spisula solidissima*, prevention of cell division by dilute solutions of cyanide is associated with a prevention of mitotic gelation. On the other hand, earlier work on the egg of the sea urchin Arbacia has been confirmed and in this egg instead of the gelation being prevented, the gelation occurs normally but the protoplasm stays in the gel state and does not return to a fluid condition as it does in the normal untreated egg. Why the protoplasm of the Arbacia egg should behave in one way and the protoplasm of the other eggs in quite a different way is not clear. But in any attempt at interpretation, it should be remembered that cyanide not only inhibits oxidizing enzymes but also acts as an accelerator of proteolytic activity; and proteolytic enzymes and clotting enzymes may be one and the same. In any event, it is hoped that further experiments may help to elucidate the relationship between oxidation and clotting phenomena in the protoplasm.

Colloidal changes during aging of the Chaetopterus egg.[1] WALTER L. WILSON AND L. V. HEILBRUNN.

Much has been written about the colloidal changes that presumably might occur in living cells when they age, but there is little definite information as to the changes that actually do occur. The fact that the cortex of a cell is especially rich in calcium and that the calcium content of cells and tissues as a whole seems to increase during senescence would make it seem probable that the cortex would become richer in calcium as cells grow older. In the egg of the worm Chaetopterus, it is possible to determine the rigidity of the cortex by relatively simple centrifuge tests (Wilson, 1951), and this rigidity seems to depend on the amount of calcium that is combined in the cortex. Soon after the egg is shed into sea water, a force of approximately 7000–9000 times gravity is required to move the granules out of the cortex of the cell. With the passage of time, there is a slow but progressive decrease in the force that is required to move the granules. Thus the rigidity of the cortex declines until some 36 hours after the egg is shed, a force of 1000 times gravity or less has the same effect in moving granules out of the cortex as much larger forces did earlier. Indeed the cortex liquefies to such an extent that the eggs tend spontaneously to disintegrate. Whether the liquefaction of the cortex is due to loss of calcium from it is not certain. However, there is an indication that calcium is released from the cortex to the cell interior. For the interior of the aging egg

[1] This investigation was supported by a research grant from the National Cancer Institute, National Institutes of Health, Public Health Service, administered by L. V. Heilbrunn.

cell becomes filled with what appear to be vacuoles, and such vacuolization may well be due to a calcium-induced surface precipitation reaction. But there is some doubt as to whether the structures observed in the aging egg are really vacuoles, for there is a possibility that they may be nuclei. This can only be determined by cytological studies of sectioned material and such studies have not yet been made.

The effect of strong centrifugal force on the development of Chaetopterus eggs.[1] WALTER L. WILSON AND YONYNAH SCHUB.

Years ago, Lillie found that when eggs of the worm Chaetopterus were centrifuged and then fertilized, development proceeded normally. However, in Lillie's experiments the centrifugal forces used were not strong enough to dislodge the granules from the cortex of the egg, where they are present normally in a single layer. It is possible therefore that the organization of the egg depends on the arrangement of these cortical granules. Wilson (1951) showed that a force of approximately 9000 times gravity, acting for one minute, was powerful enough to move the granules from the cortex. In such eggs development does not proceed normally. Whether the effect of strong centrifugation is due to a movement of the granules or to a weakening of the cortical layer is not yet certain. We have found that forces up to 2600 times gravity, acting for one minute, do not affect normal development. At forces as high as 3500 times gravity, acting for one minute, a few of the eggs developed into motile larvae. None does so following centrifugation for a minute at forces 4300 times gravity. These forces are sufficient to dislodge some but not all of the cortical granules.

A globulite structure for acid insulin sulphate?[2] DOROTHY WRINCH.

In a preliminary study of the dry orthorhombic acid insulin sulphate crystal, Low (1950–3) has recorded principal vector projections and two vector sections. A first question regarding the insulin entities in this crystal, entities with M.W. ca. 11,700 and 12 sulphate groups apiece, is the nature of the polymers of amino acid backbone monomers, N-C_α-C, which form their molecular skeletons. To obtain information as to whether these skeletons are uniaxial, biaxial or genuinely three-dimensional in nature, the vector maps are examined in detail in the neighborhood of the origin. We find no indication of some one particular direction, in or perpendicular to which the distribution of short inter-atomic vectors has special significance. It may be concluded that, for this protein, as for horse hemoglobin and for ribonuclease (Wrinch 1952, 1953), the skeletons are genuinely three-dimensional in their structure and the traditional type of structure comprising a bundle of polypeptide chains may be rejected at the outset. Proceeding with the examination of the vector maps further from the origin, indications are seen, in the low density regions in many directions preceding the high density regions in many directions at about 9–11 Å from the origin, that there may be two globulite structures (separate or associated) in each of these insulin entities, in accordance with the suggestions made regarding trigonal insulin (Wrinch 1948, 1952) in the light of Crowfoot's X-ray data (1946).

LALOR FELLOWSHIP REPORTS

Isolation of protein fractions from echinoderm sperms and eggs. D. HAMER.

Specimens of basic nuclear proteins have been prepared from isolated sperm nuclei of Arbacia, Echinarachnius and Asterias for amino acid analysis. Nuclei were isolated in a saline-citrate medium and then extracted directly with 0.2 *M* HCl. The acid extracts were dialysed and the protein precipitated as the picrate. The picrates were almost completely

[1] This investigation was supported by a research grant from the National Cancer Institute, National Institutes of Health, administered by L. V. Heilbrunn.

[2] This work is supported by the ONR under contract with Smith College.

soluble in acetone and from the solutions the basic proteins were obtained as the sulphate by addition of a few drops of 2 *M* sulphuric acid. (Only in the case of Asterias sperm was there an appreciable amount of insoluble picrate.) Similar basic fractions are obtained by acid extraction of whole nucleo-protein prepared using strong saline solutions. As is the case with mammalian cells, there was an appreciable amount of protein firmly bound to the nucleic acid and so specimens were also prepared for estimations of the amounts of the major components (nucleic acid, basic protein and acid-insoluble protein).

Preparation of nuclear fractions from unfertilized eggs is a more difficult problem due to the large amounts of non-nuclear material present. Some fractions were obtained, however, by direct extraction of the eggs followed by fractionation of the extracts using the picrate technique described above. In one case (Arbacia) it was possible to prepare corresponding fractions from fertilized eggs. Analysis of this protein may give information about the stage at which the protamine-type basic protein develops into the more complicated histone molecule.

When the amino acid analyses are completed it will be interesting to see if species differences are reflected by variations in the composition of the nuclear proteins, since these are believed to be constituents of the chromosomes.

Oxidation of amino acids by marine animals. D. Hamer.

D-amino acid oxidase has been found to be widely distributed in mammals, particularly in the liver and kidney. Recently other workers have demonstrated for the first time the occurence of this oxidase in invertebrates using Octopus and Sepia.

It has been found that the squid, *Loligo pealii*, contains in the liver an enzyme system capable of oxidizing D-amino acids. Homogenates of the liver were made in 0.5 *M* sodium chloride and the oxidation of amino acids was studied at 25° C. and pH 7.4 using the usual constant volume manometric procedures. The enzyme system was inactivated rapidly at 37° and pH 7.4 and particularly at pH 8.4 which is usually used for determinations on mammalian tissues. The amino acids were used as the DL-forms at a final concentration of 0.02 *M*. Rate of amino acid oxidation was measured as the excess oxygen uptake beyond that due to the endogenous respiration and was expressed as μl. oxygen consumed/hour/mg. homogenate nitrogen. Alanine was oxidized most rapidly of the compounds studied, the mean observed rate being 4.65. Oxidation of leucine and methionine was less rapid and that of phenylalanine still lower (2.8, 2.6, and 1.6, respectively).

Under the same conditions the liver of the smooth dogfish, *Mustelus canis*, oxidized alanine about three times as fast as the squid (12.0 μl/hr./mg. N) but no significant uptake was detected using the other amino acids which were oxidized by Loligo. No oxidation of any of these amino acids was found using homogenates of digestive organs of the starfish (*Asterias forbesi*).

Observations with an interference microscope. A. F. Huxley.

The range of usefulness of a two-beam interference microscope, designed and built for quantitative work on isolated cells, was explored by making measurements on a variety of specimens, most of which were provided by other investigators in the Laboratory. The microscope has a water-immersion objective (n.a. 0.9), and the retardation of light by the specimen (nearly proportional to dry weight per unit projected area) can be compensated and thus measured.

1. *Human erythrocytes.* Red cells suspended in heparinized plasma were photographed with increasing amounts of compensation. A satisfactory picture of the shape of the cells was obtained from these "contour" photographs, together with refractometer measurements, and the mean cell volume agreed with that obtained from the haematocrit and cell count.

2. *Nucleoli from Asterias eggs.* Useful estimates of the solid content of nucleoli isolated by Dr. Vincent were obtained, but their precision was limited by the irregular vacuolation of these structures.

3. *Nuclei of Arbacia eggs.* The solid content of nuclei isolated by Dr. Tsuboi was estimated, and was found to confirm an observation by him on their specific gravity.

4. *Phascolosoma muscle.* Photographs were taken of muscle cells isolated by Dr. Prosser by maceration of the retractor muscles of the proboscis of *Phascolosoma*.

5. *Isolated myofibrils.* Fibrils obtained by treating glycerinated rabbit muscle in the Blendor were examined in collaboration with Dr. A. G. Szent-Györgyi. The extraction of various components of the striations by salt solutions was observed.

The effects of various acids on the receptors of the amphibian tongue. THOMAS N. JOHNSON.

Sensory impulses were recorded from the frog glossopharyngeal nerva. The impulses were excited by application of water, sea water, and mechanical stimulation to the sensory receptors in the tongue. Solutions of three acids, acetic, citric, and hydrochloric, were applied to the tongue, and the stimulation repeated. Buffered solutions of each acid at pH 6.0, 5.5, 4.5, and 3.5 were employed.

At pH 6.0, none of the acids affected the sensory discharges.

At pH 5.5, application for three minutes of acetic and of hydrochloric acid diminished the responses to all stimuli. After return from the acid to amphibian Ringer, the responses increased, but not to their original level. Further application of these acids completely and irreversibly abolished responses to water and sea water, but responses to touch remained.

At the same pH (5.5), citric acid had the reverse effect. The responses to all stimuli were increased for several minutes after application. The enhancement by citric acid is counteracted by the addition of acetic acid as well, which retains its effect of diminishing responses below the normal level.

At pH 4.5, all three acids diminish the responses, and a second application abolishes them.

At pH 3.5, all acids employed abolish responses to water and sea water, but responses to touch persist for as long as two hours.

It appears that moderate H ion concentrations diminish responses, and that high H ion concentration irreversilbly damages the sensory apparatus. Citrate ions increase the responses, probably by the precipitation of calcium ions, and this increase masks the depression by the H ions, provided that the H ion concentration is moderate. At the high H ion concentrations, the depression becomes predominant.

A study of an unidentified ultraviolet absorbing substance occurring in the tissues of marine animals. HERMAN M. KALCKAR, JACK L. STROMINGER AND NORMAN R. GEVIRTZ.

The mantle tissue and the foot muscle of *Busycon canaliculatum* were found to contain large amounts of a substance with maximum absorption at 272 mμ. The absorption spectrum does not change between pH O and 14 and is unaffected by treatment with sodium nitrite or by heating to about 200° C. in concentrated HCl. The compound was prepared by absorbing partially neutralized (pH 4 to 5) perchloric acid extracts of tissue on Norite and eluting with 50 per cent slightly ammoniacal ethanol. A further purification step by which all ninhydrin-positive material was removed consisted of saturation with Na_2CO_3, repeated extractions with 5 volumes of water-saturated butanol and finally returning the compound to aqueous solution by extraction with water. At this point an impurity can be precipitated with Reinecke salt at pH 1. The compound has the following properties: contains nitrogen; R_f in ammoniacal butanol 0.12, in 5 per cent KH_2PO_4 saturated with isoamyl alcohol 0.85; immobile on paper electrophoresis at pH 7.9; absorbed on Dowex-50-H^+ and eluted with 2 N HCl; not retained on Dowex-2-acetate even at pH 12. As judged by most of the above listed properties the same compound has been obtained from the muscle of lobster and crab, and also from the nerve tissue of crab and lobster and from squid axoplasm (*cf.* A. Shanes, 1951). The possible identity with or relationship to homarin, *i.e.*, N-methyl picolinic acid betaine (*cf.* F. Hoppe-Seyler, 1933), is under investigation.

The bacterial reduction of nitro compounds. JOSEPH R. MERKEL.

Bacteria were selected which could reduce nitrate to various levels including ammonia and gaseous nitrogen. The bacteria used included laboratory strains and strains isolated from infected marine specimens. All the strains had the ability to reduce nitrates to nitrites, and most

of the cultures produced large amounts of ammonia and hydrogen. The production of hydrogen and ammonia tended to obscure any production of nitrogen.

Manometric studies with strain B of *Escherichia coli* and with a strain of *Proteus* (presumably, *vulgaris*) indicated that the production of ammonia from nitrate or nitrite involved the formic "hydrogenlyase" system. Addition of nitrate or nitrite to a phosphate-buffered medium (pH. 6.9) containing formate or glucose and inorganic salts, under anaerobic conditions, caused a reduction in the rate of hydrogen production which was equivalent to the amount of nitrate or nitrite converted to ammonia. The decrease in the rate of gas production may be a result of the hydrogenase activity of these cultures; however, it seems more likely that the reduction of the nitro compounds is linked more directly to the formic dehydrogenase system. In this system the reduction of nitrate is analogous to reduction by zinc and HCl, where the nascent hydrogen is picked up by an oxidized substance before molecular hydrogen is formed, rather than an activation of gaseous hydrogen.

A possible method which has been suggested for the formation of nitrogen by bacterial cultures (denitrification) is through the reaction of nitrous acid with amino compounds. It is conceivable, on the basis of the relative ease of reduction of aromatic nitro compounds, that this reaction may be mediated through aromatic nitro and amino compounds. Future experiments with active denitrifying cultures will be designed to test this hypothesis.

Nucleic acid and protein distribution in unfertilized and fertilized eggs of Arbacia. K. K. Tsuboi.

Homogenates of Arbacia eggs were subjected to successive centrifugal forces ranging from 250–100,000 × g and the resulting fractions examined for nucleic acids and protein (protein estimated by nitrogen, nucleic acids by u. v. absorption and phosphorus following appropriate fractionation).

Unfertilized eggs homogenized in isotonic, hyper- and hypotonic electrolyte solutions (KCl, NaCl, artificial sea water) and non-electrolytes (sucrose, dextrose) in the presence and absence of added chelating agents (citrate, Versene) were investigated. The presence of chelating agents in homogenates prepared in hypotonic electrolyte solutions and all non-electrolyte solutions resulted in a redistribution of the bulk of cytoplasmic pentose nucleic acid (PNA) from a form sedimentable only at very high centrifugal force (100,000 × g) to a relatively non-sedimentable state. The homogenization of eggs in stronger salt solutions (> 0.4 *M* KCl) only, resulted also in a "solubilization" of the PNA. Although some redistribution of protein was apparent in these studies, the extent was not comparable to that found for the PNA. PNA was readily sedimentable from homogenates prepared in artificial sea water. From these results it is concluded that the PNA in unfertilized eggs is present in submicroscopic particulates in combination with calcium and/or magnesium as a binding force in maintenance of the complex.

Experiments on fertilized Arbacia eggs are only of a preliminary nature due to difficulties encountered in preparing suitable homogenates from the embryos. Little evidence was found in preliminary experiments for the presence of extranuclear DNA within 20 hour embryos. A redistribution of protein and PNA appeared to be present within the 20-hour embryo, resulting in a much greater proportion of sedimentable protein at 100,000 × g and a more ready sedimentation of PNA within fractions obtained by less centrifugal force.

The apyrase activity of mantle muscle of Loligo pealii. G. W. de Villafranca.

An investigation of the apyrase activity of squid mantle muscle was undertaken in an attempt to ascertain whether this muscle contained the two apyrases, *i.e.*, particulate and myosin, found in rat and locust muscle.

Squid mantle was extracted with distilled water, molar sucrose, or 0.05 *M* KCl. After the water or sucrose extraction the residue was re-extracted with 0.5 *M* KCl to recover the myosin proteins. Myosin was precipitated from the 0.5 *M* KCl extract by dilution with 6 volumes of distilled water and was re-precipitated once or twice. The apyrase reaction was carried out at 25° C. in 0.005 *M* KCl and 0.002 *M* ATP under conditions where the activity was directly proportional to the concentration of protein and to the time incubated. The

myosin activity was maximal at pH 5.8 and 0.01–0.03 *M* $CaCl_2$ yielding Q_P values as high as 1700. $MgCl_2$ gave slight activation at concentrations of 0.01–0.05 *M* while $CaCl_2$, in optimal concentrations, gave 8–10 fold activation. A K_m of 4.07×10^{-4} was obtained.

Under the extraction conditions tried, the major portion of the total apyrase activity (60–80%) was recovered with the myosin fractions. Another 10% of the activity was recovered in material sedimented at 20,000 g which had the same pH optimum and ion activation as the myosin fractions. At least 70% of this activity, however, could be removed by 0.5 *M* KCl. It would seem that the apyrase of large particles from squid mantle is negligible or absent. Less than 2% of the total activity was found in fractions sedimenting at 100,000 g.

It appears, therefore, that squid mantle muscle has only one apyrase associated, apparently, with the myosin proteins. Preliminary experiments indicate that a similar situation exists in muscle from *Mustelus canis*, although here the apyrase is Mg^{++} activated.

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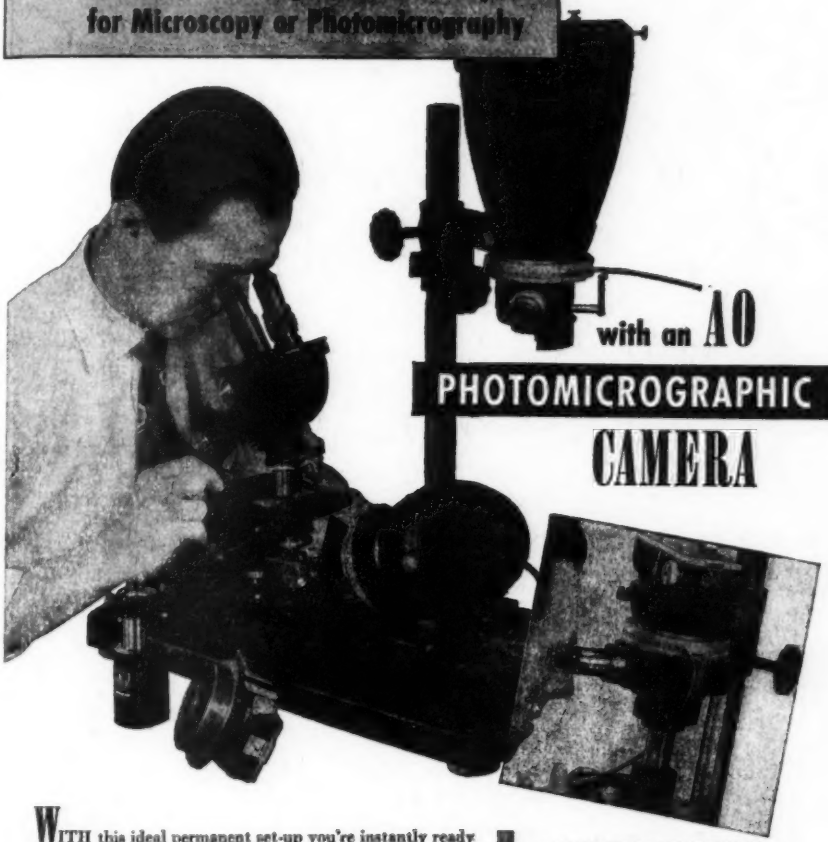

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